PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : G01N 27/327, 33/543, C12Q 1/00, G06F 19/00 // 159:00

A1

(11) International Publication Number: **WO 99/63333**

(43) International Publication Date: 9 December 1999 (09.12.99)

(21) International Application Number: PCT/SE99/00921

(22) International Filing Date: 31 May 1999 (31.05.99)

(30) Priority Data:
09/087,402 29 May 1998 (29.05.98) US

(71) Applicant *(for all designated States except US)*: BIACORE AB [SE/SE]; Rapsgatan 7, S–754 50 Uppsala (SE).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: ANDERSSON, Karl [SE/SE]; Ulleråkersvägen 62, S–756 43 Uppsala (SE). HÄMÄLÄINEN, Markku [SE/SE]; Björkkulla Funbo, S–755 97 Uppsala (SE). MALMQVIST, Magnus [SE/SE]; Rörbäcksvägen 39, S–757 57 Uppsala (SE). ROOS, Håkan [SE/SE]; Oslogatan 85, S–752 64 Uppsala (SE).

(74) Agent: AWAPATENT AB; P.O. Box 45086, S–104 30 Stockholm (SE).

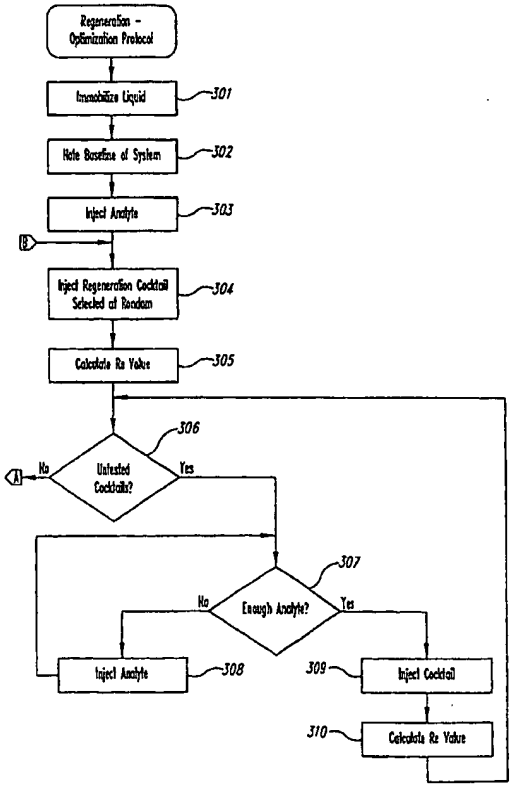
(81) Designated States: AU, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: SURFACE REGENERATION OF BIOSENSORS

(57) Abstract

Surface regeneration of affinity biosensors and characterization of biomolecules associated therewith by multivariate technique employing cocktails of regeneration agents to optimize regeneration of biosensor surface and/or characterize biomolecules associated therewith. Kits and stock solutions for use in the context of this invention, as well as associated computer algorithms are also disclosed.



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

SURFACE REGENERATION OF BIOSENSORS

TECHNICAL FIELD

This invention relates generally to surface regeneration of affinity biosensors and characterization of biomolecules associated therewith and, more particularly, to use of a multivariate technique employing cocktails of regeneration agents to optimize regeneration of the surface of a biosensor and use of a related chemical perturbation technique to characterize biomolecules associated therewith, as well as related regeneration kits, reagents, algorithms and communicative methods for the same.

BACKGROUND OF THE INVENTION

A variety of analytical techniques are used to characterize interactions between molecules, particularly in the context of assays directed to the detection and interaction of biomolecules. For example, antibody-antigen interactions are of fundamental importance in many fields, including biology, immunology and pharmacology. In this context, many analytical techniques involve binding of a "ligand" (such as an antibody) to a solid support, followed by contacting the ligand with an "analyte" (such as an antigen). Following contact of the ligand and analyte, some characteristic is measured which is indicative of the interaction, such as the ability of the ligand to bind the analyte. After measurement of the interaction, the ligand-analyte pair must be disrupted in order to "regenerate" free ligand for a further analytical measurement.

A number of techniques have been employed to regenerate surface-bound ligands. Most commonly, regeneration involves a series of trial and error attempts to remove the analyte from the ligand, while minimizing loss of ligand from the solid support. Care must also be taken not to use a regeneration solution that is too aggressive in order to avoid partial or complete loss of ligand activity. Furthermore, regeneration must not influence the ligand with regard to subsequent measurements, otherwise results from assay-to-assay will not be truly comparable. These problems may be avoided by simply discarding the solid support after each assay. However, this is undesirable since generation of the solid support having bound ligand can be both costly and time consuming, and very often the researcher has only limited quantities of the ligand and/or solid support. Accordingly, improved techniques for regenerating such surfaces are desired.

The need to effectively regenerate a solid surface may be illustrated in the context of biosensors which use surface plasmon resonance (SPR) to monitor the interactions between an analyte and a ligand bound to a solid support. In this regard, a representative class of biosensor instrumentation is sold by Biacore AB (Uppsala, Sweden) under the trade name BIAcore® (hereinafter referred to as "the BIAcore instrument"). The BIAcore instrument includes a light emitting diode, a sensor chip covered with a thin gold film, an integrated fluid cartridge and photo detector. Incoming light from the diode is reflected in the gold film and detected by the photo detector. At a certain angle of incidence ("the SPR angle"), a surface plasmon wave is set up in the gold layer, which is detected as an intensity loss or "dip" in the reflected light.

The SPR angle depends on the refractive index of the medium close to the gold layer. In the BIAcore instrument, dextran is typically coupled to the gold surface, and a ligand is bound to the dextran layer. The analyte of interest is injected in solution form onto the sensor surface through a fluid cartridge. Since the refractive index in the proximity of the gold film depends upon (1) the refractive index of the solution (which is constant) and, (2) the amount of material bound to the surface, the interaction between the bound ligand and analyte can be monitored as a function of the change in SPR angle.

A typical output from the BIAcore instrument is a "sensorgram," which is a plot of response (measured in "resonance units" or "RU") as a function of time. An increase of 1000 RU corresponds to an increase of mass on the sensor surface of approximately 1 ng/mm^2. As sample containing an analyte contacts the sensor surface, the ligand bound to the sensor surface interacts with the analyte in a step referred to as "association." This step is indicated on the sensorgram by an increase in RU as the sample is initially brought into contact with the sensor surface. Conversely, "dissociation" normally occurs when sample flow is replaced by, for example, a buffer flow. This step is indicted on the sensorgram by a drop in RU over time as analyte dissociates from the surface-bound ligand.

A representative sensorgram for the BIAcore instrument is presented in Figure 1, which depicts an antibody surface interacting with analyte in a sample. During sample injection, an increase in signal is observed due to binding of the analyte (*i.e.*, association) to a steady state condition where the resonance signal plateaus. At the end of sample injection, the sample is replaced with a continuous flow of buffer and decrease in signal reflects the dissociation of analyte from the surface. The slope of the association/dissociation curves provide valuable

information regarding the reaction kinetics, and the height of the resonance signal represents surface concentration (*i.e.*, the response resulting from an interaction is related to the change in mass concentration on the surface). While dissociation will naturally tend to regenerate some portion of the sensor surface, only a very small portion of the sensor surface is typically regenerated in this manner, especially when their is a strong interaction between the ligand and analyte. Thus, some further regeneration step is often needed in order to effectively remove analyte from the sensor surface and ready the surface for contact with a new sample.

Numerous articles have been published directed to the use of the BIAcore instrument in the analysis of biomolecular interactions. In these articles, researchers have reported a variety of regeneration agents and techniques for regenerating the sensor surface prior to contact with a new sample. In general, these articles had main goals other than surface regeneration; however, three papers discussed systematic investigations of regeneration practices concerning antibody-antigen assays (Brigham & O'Shannessy, *Chromatographia 35*:45-49, 1993; Brigham et al., *Analytical Biochemisty 205*:125-131, 1992; Minunni et al., *Analytical Letters 26*:1441-60, 1992), with perhaps the most extensive treatment being that of Burke & O'Shannesy (1993). In that reference, a sCR1-MAb YZ1 system was regenerated using various regeneration agents. The results of this study indicated that, among several common regeneration agents, only a few had a high regeneration effect for the sCR1-MAb YZ1 system. The authors reported that the choice of acid can be more important than the choice of pH (*e.g.*, 0.1M phosphoric acid, pH 1.3, worked better than 0.1M HCl, pH 1.0), and that combinations of agents in some cases are favorable (*e.g.*, 50% ethyleneglycol/0.1M triethylamine, pH 10.5, was more favorable than 0.1M triethylamine, pH 10.5).

More generally, the above-noted articles disclose that various classes of ligand-analyte systems may be regenerated under the following conditions:

Antibody-antigen assays - to varying degrees with hydrochloric acid (HCl) of different concentrations (Malmborg et al, *Scandinavial Journal of Immunology 35*:643-50, 1992; Ward et al., *Biochemistry International 26*:559-65, 1992) or with weaker acids, typically phosphoric or formic (Corr et al., *Journal of Experimental Medicine 178*:1877-92, 1993; VanCott et al., *Journal of Immunological Methods 183*:103-17, 1995), or with detergent or chaotropic solutions (Tanchou et al., *AIDS Research and Human Retroviruses, 10*:983-93 1994; End et al., *Journal of Biological Chemistry 268*:10066-75, 1993);

Receptor-transmitter assays - with acids (Morelock et al., *Journal of Medicinal Chemistry 38*:1309-18, 1995), bases (Lemmon et al., *Journal of Biological Chemistry 269*:31653-58, 1994), under chaotropic conditions and high ion strength (Stitt et al., *Cell 80*:661-70, 1995), or under natural dissociation conditions (Ma et al., *Journal of Biological Chemistry 39*:24430-36, 1994);

Assays containing DNA - under very mild regeneration conditions using detergents, EDTA, or under natural dissociation conditions (Cheskis et al., *Molecular Endocrinology* 1996; Casasnovas *Journal of Biological Chemistry 270*:13216-24, 1995); and

Assays containing glycoproteins - under acid conditions or using sugar solutions (Okazaki et al., *Journal of Molecular Recognition 8*:95-99 1995).

While these articles disclose a variety of regeneration techniques, those techniques are system dependent and are not particularly effective beyond the parameters of the specific system reported in each paper. Thus, anytime a researcher investigates a new ligand-analyte system, a great deal of time and effort may be spent identifying regeneration conditions suitable for the system at hand, often with varying degrees of success. Accordingly, there is a need in the art for improved techniques for regenerating the surface of an affinity biosensor.

There is also a need in the art for techniques to characterize the analyte and/or ligand associated with the surface of an affinity biosensor. Such characterization can occur either prior to the regeneration of the biosensor surface (*e.g.*, during association or dissociation) or can occur during regeneration. Further, the ability to predict structure-activity relationships ("SAR") has become an important goal in a variety of fields. For example, as the number of known protein structures has increased, researchers have tried, with limited success, to predict SAR for such proteins. In the context of monoclonal antibodies, one goal has been to design a MAb that binds specifically to a given antigen, in advance of laboratory experiments. Accordingly, a need exists for techniques that can predict SAR for new analytes and/or ligands, such as proteins, and thus characterize their activity in advance of laboratory analysis of the same.

There is also a need to characterize analytes and/or ligands with respect to changing chemical environments. For example, in developing quantitative assays for determination of vitamin concentration in food, researchers are often interested in knowing how sensitive a specific molecule, typically a MAb, is to variations in its chemical environment. An aqueous solution having a known amount of vitamin, for example, may be more sensitive than a crude sample (*e.g.*, infant

formulas, cereals, etc.) having the same concentration of vitamin. Therefore, the measured concentration of vitamin from the crude sample may be different than its true concentration. Similarly, when determining drug and/or hormone residues in animals (*e.g.*, in urine), researchers are also often interested in knowing how sensitive a specific molecule is to variations in its chemical environment. Accordingly, a need exists for techniques that can predict the performance of a specific molecule in a crude sample. Such techniques may also be useful in developing quantitative assays.

Furthermore, there is also a need in the art to detect and characterize minor structural differences in, for example, proteins. Researchers often desire to verify that manufactured proteins have their expected structures, and are not point-mutated or post modified (*i.e.*, by substitutions by carbohydrates, fatty acids, *etc.*). Accordingly, a need exists for methods useful for detecting minor structural differences in proteins.

In addition to the foregoing, there is also a need to effectively describe and communicate the changing nature of biomolecular interactions as the surrounding chemical environment is perturbed. That is, researchers who are interested, for example, in knowing the stability of a biomolecular interaction in various chemical environments, need a method for obtaining such information.

The present invention fulfills these needs, and provides further related advantages.

SUMMARY OF THE INVENTION

In brief, the present invention is directed to regeneration of a biosensor surface, particular with regard to identification and selection of regeneration agents and conditions for use of the same, as well as to the characterization of biomolecules associated with the biosensor surface. Reagent kits and methods are also disclosed for use within the context of this invention.

In one embodiment, a method is disclosed for selecting an optimized regeneration solution for the regeneration of a biosensor surface having a surface-bound ligand and an analyte associated with the ligand. The method comprises the steps of:

(a) sequentially contacting the biosensor surface with each of a plurality of first regeneration cocktails, wherein each of said first regeneration cocktails is an aqueous solution comprising at least one acidic, basic, ionic, organic, detergent or chelating stock solution, and wherein at least one of said first regeneration cocktails comprises a mixture of at least two of said stock solutions;

(b) measuring the regeneration effect for each of said plurality of first regeneration cocktails to determine which of said plurality of first regeneration cocktails have the highest measured regeneration effect;

(c) selecting at least two different stock solutions present in said plurality of first regeneration cocktails having the highest measured regeneration effect;

(d) combining said at least two different stock solutions in various ratios to generate a plurality of second regeneration cocktails;

(e) sequentially contacting the biosenor surface with each of said plurality of second regeneration cocktails; and

(f) determining the regeneration effect of each of said plurality of second regeneration cocktails and therefrom identifying a second regeneration cocktail as the optimized regeneration solution.

In a further aspect of this embodiment, the method further comprising, after step (f), the following additional steps:

(g) combining said at least two different stock solutions in different ratios than step (d) to generate a plurality of third regeneration cocktails;

(h) sequentially contacting the biosensor surface with each of said plurality of third regeneration cocktails; and

(i) determining the regeneration effect of each of said plurality of third regeneration cocktails and therefrom identifying a third regeneration cocktail as the optimized regeneration solution.

The above steps may be repeated until the optimized regeneration solution is identified.

In another embodiment of this invention, a reagent kit is disclosed containing at least two different stock solutions for use within the above method. Preferably, all six different stock solutions are present in the reagent kit - that is, an acidic stock solution, a basic stock solution, an ionic stock solution, an organic stock solution, a detergent stock solution, and a chelating stock solution.

In still a further embodiment, a computer system is disclosed for selecting an optimized regeneration solution for the regeneration of a biosensor surface having a surface-bound ligand and an analyte associated with the ligand. The computer systems performs the following steps:

(a) instructing a device to combine a series of stock solutions in various ratios to generate a plurality of first regeneration cocktails;

(b) sequentially controlling the device to contact the biosensor surface with each of said plurality of first regeneration cocktails;

(c) determining the regeneration effect of each of said first regeneration cocktails on the biosensor surface based on measurements received from the device;

(d) selecting at least two different stock solutions having the highest regeneration effect;

(e) instructing the device to combine a subset of said at least two different stock solutions in varying ratios to generate a plurality of second regeneration cocktails;

(f) sequentially controlling the device to contact the biosenor surface with each of said second regeneration cocktails; and

(g) determining the regeneration effect of each of said second regeneration cocktails based on measurements received from the device, and therefrom identifying a second regeneration cocktail as the optimized regeneration solution.

In a further embodiment, a method is disclosed for characterizing a ligand and/or analyte associated with a biosensor surface, comprising the steps of:

(a) sequentially contacting the biosensor surface having a surface-bound ligand with each of a plurality of characterization solutions, wherein each of said characterization solutions is an aqueous solution comprising at least one acidic, basic, ionic, organic, detergent or chelating solution;

(b) introducing the analyte into each of said plurality of characterization solutions so as to interact the analyte with the surface-bound ligand;

(c) measuring at least one of the association rate, surface-bound analyte concentration, and dissociation rate of the analyte-ligand interaction for each of said plurality of characterization solutions; and

(d) characterizing the ligand and/or analyte associated with the biosensor surface based on at least one of the association rate, surface-bound analyte concentration, dissociation rate, and regeneration effect of the analyte-ligand interaction for each of said plurality of characterization solutions.

In a further aspect of this embodiment, the method further comprises, after step (d), the following additional step:

(e) comparing the characterization of the ligand and/or analyte associated with the biosensor surface with a set of predetermined characterizations of

other test molecules, and thereby predicting the activity of the ligand and/or analyte associated with the biosensor surface.

In still a further embodiment, a computer-readable medium is disclosed containing the instructions for performing the above methods.

In yet a further embodiment, a computer memory containing a data structure useful for communicating chemical perturbation information associated with the analyte-ligand interaction is disclosed (as well as a generated data signal conveying the same). The data structure comprises one or more kinetic parameters wherein each kinetic parameter expressed in terms of a mathematical model that describes the relation between the kinetic parameter and the analyte-ligand interaction in a plurality of characterization solutions, such that the data structure may be used to communicate chemical perturbation information associated with the analyte-ligand interaction.

These and other aspects of this invention will be evident upon reference to the attached drawings and following detailed description. To this end, various references are cited throughout this application to further illustrate specific aspects of this invention. Such documents are each incorporated herein by reference in their entirety.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a representative sensorgram illustrating association, steady-state, dissociation and regeneration of a biosensor surface.

Figure 2 shows a two-dimensional mixture optimization design ("MO2D"), wherein the compositions of 10 different two-stock cocktails are denoted.

Figure 3 shows a three-dimensional mixture optimization design ("MO3D"), wherein the compositions of seven different three-stock cocktails are denoted.

Figure 4A and Figure 4B provide a flowchart illustrating the steps for identifying and optimizing the regeneration conditions for a biosensor surface.

Figure 5 shows a sensorgram from a regeneration-optimization protocol run with a p24/MAb 609 system.

Figure 6 illustrates the measured R_e values for a plurality of screening cocktails tested on a p24/MAb 609 system.

Figure 7A shows an overlay plot of the sensorgrams of the first and the twentieth injection of analyte p24 onto a MAb 609 surface in an experiment where

one injection of analyte followed by one injection of AIC regeneration cocktail were performed twenty times.

Figure 7B shows the difference between the sensorgrams of the first and the twentieth injection of analyte p24 onto the MAb 609 surface of Figure 7A, and demonstrates that the 19 regenerations have not influenced the surface in any permanent sense.

Figure 8 illustrates the measured R_e values for a plurality of screening cocktails tested on a p24/MAb 576 system.

Figure 9 illustrates the measured R_e values for a three-dimensional mixture optimization design ("MO3D") experiment on a p24/MAb 576 system, wherein the regeneration cocktails include combinations of acidic (A), ionic (I), and chelating (C) stock solutions.

Figure 10 illustrates the measured R_e values for a plurality of screening cocktails tested on a GST-polyclonal antiGST system.

Figure 11 illustrates the measured R_e values for a two-dimensional mixture optimization design ("MO2D") experiment on a GST/polyclonal antiGST system, wherein the regeneration cocktails include combinations of acidic (A) and ionic (I) stock solutions, and wherein the volume percent and acidity of the acidic (A) stock were varied and the ionic (I) stock included 0.5M EDTA.

Figure 12 illustrates the measured R_e values for a plurality of screening cocktails tested on a biosensor having a bound ligand, SpA, with the following MAb analytes: g3_clone2, g3_clone3, g2b_clone2, g2b_clone3, g2a, gla, anti-theofyllin MAb 459, anti-p24 MAb 609, polyclonal anti-clenbuterol Abs r200 and rl54.

Figure 13 shows a high-level block diagram of a exemplary computer system for communicating chemical perturbation information.

Figure 14 shows the average change of k_a and k_d when changing pH, NaCl(Na), EDTA (ED), KSCN (SCN), and DMSO (DM) from a low to high level in accordance with Example 2.

Figure 15 shows a diagram summarizing the effects of increasing the designated parameter in accordance with Example 2.

Figure 16 shows overlaying sensorgrams from the buffers giving the highest and lowest affinity in accordance with Example 2.

Figure 17 shows the average change of k_d when changing pH, NaCl(Na), EDTA (ED), KSCN (SCN), and DMSO (DM) for the interaction of IgG3 with Z6G in accordance with Example 3.

Figure 18 shows the average change of k_d when changing pH, NaCl(Na), EDTA (ED), KSCN (SCN), and DMSO(M) for the interaction of IgG3 with Z in accordance with Example 3.

Figure 19a shows artificial buffer samples spiked with biotin at four concentrations and run with antibiotin from Novocastra in accordance with Example 4.

Figure 19b shows artificial buffer samples spiked with biotin at four concentrations and run with antibiotin from Goldmark in accordance with Example 4.

Figure 19c shows artificial buffer samples spiked with biotin at four concentrations and run with antibiotin from Sigma in accordance with Example 4.

Figure 19d shows artificial buffer samples spiked with biotin at four concentrations and run with antibiotin from Pierce in accordance with Example 4.

Figure 20a shows infant formula samples spiked with biotin at three concentrations and run with antibiotin from Novocastra in accordance with Example 4.

Figure 20b shows infant formula samples spiked with biotin at three concentrations and run with antibiotin from Goldmark in accordance with Example 4.

Figure 20c shows infant formula samples spiked with biotin at three concentrations and run with antibiotin from Sigma in accordance with Example 4.

Figure 20d shows infant formula samples spiked with biotin at three concentrations and run with antibiotin from Pierce in accordance with Example 4.

Figure 21 shows sum of standard deviation in infant formula samples plotted vs. sum of standard deviation in artificial buffer in accordance with Example 4, wherein raw data was used for the calculation.

Figure 22 shows sum of standard deviation in infant formula samples plotted vs. sum of standard deviation in artificial buffer in accordance with Example 4, wherein normalized data was used for the calculation.

Figure 23 shows a plot of the kinetic parameters k_a and k_d measured in HBS buffers in accordance with Example 5.

Figure 24 shows a plot of the relative perturbation of k_a by NaCl and DMSO in accordance with Example 5.

Figure 25 shows a plot of the relative perturbation of k_d by NaCl and DMSO in accordance with Example 5.

Figure 26 shows overlay sensorgrams from four different peptides interacting with FAb 57p, wherein the sensorgrams were normalized to the binding level for DRK (FAb concentration 154-160nm).

Figure 27a shows measured relative perturbation vs. predicted relative perturbation for k_a perturbed by DMSO in accordance with Example 5.

Figure 27b shows measured relative perturbation vs. predicted relative perturbation for k_a perturbed by NaCl in accordance with Example 5.

Figure 27c shows measured relative perturbation vs. predicted relative perturbation for k_d perturbed by DMSO in accordance with Example 5.

Figure 28a shows normalized coefficients for the mathematical model relating peptide sequence to DMSO perturbations on k_a in accordance with Example 5.

Figure 28b shows normalized coefficients for the mathematical model relating peptide sequence to NaCl perturbations on k_d in accordance with Example 5.

Figure 28c shows normalized coefficients for the mathematical model relating peptide sequences to DMSO perturbations on k_d in accordance with Example 5.

Figure 29 shows the sensitivity of k_d to variations of the NaCl concentration for different settings of the descriptors 145 and the sum_c in accordance with Example 5.

Figure 30 shows relative buffer perturbation for the peptides DYD, DRK and DSA in accordance with Example 5.

Figure 31a shows a sensorgram of human myoglobin (dashed line) (544 nM) and sheep myoglobin in buffer P1 in accordance with Example 6.

Figure 31b shows a sensorgram of human myoglobin (dashed line) (544 nM) and sheep myoglobin in buffer P9 in accordance with Example 6.

Figure 32a shows a sensorgram of human myoglobin (dashed line) (544 nM) and sheep myoglobin in buffer P1 in accordance with Example 6.

Figure 32b shows a sensorgram of human myoglobin (dashed line) (544 nM) and sheep myoglobin in buffer P9 in accordance with Example 6.

Figures 33a-c show plots from all three experiments of D1 vs. D2-D1 for human myoglobin binding to MAb 8e11 and 22:3 in accordance with Example 6.

DETAILED DESCRIPTION OF THE INVENTION

As mentioned above, this invention is directed to regeneration of a biosensor surface, particular with regard to identification and selection of regeneration agents and conditions for use of the same, as well as to the characterization of biomolecules associated with a biosensor surface. This invention

also provides reagent kits for use within the context of this invention, as well as related algorithms and computer-readable mediums containing the same.

In one embodiment, a method is disclosed for selecting an optimized regeneration solution for regenerating a biosensor surface (*i.e.*, a regeneration identification and optimization (RO) protocol) having a surface-bound ligand and an analyte associated therewith. The analyte is typically bound (*i.e.*, associated) to the ligand by non-covalent forces (*e.g.*, electrostatic and Lewis acid-Lewis base forces). Suitable biosensor surfaces include a wide number of biosensors, particularly affinity-based biosensors. Such biosensors often have a binding or interaction partner bound to the surface which is to be contacted with the sample of interest. In the context of this invention, the agent bound to the surface of the biosensor (*e.g.*, the biosensor's sensing surface) is referred to as a "ligand," while the interactant in solution (*e.g.*, the sample) is called the "analyte."

As used herein, the terms "ligand" and "analyte" are to be construed broadly, and encompass a wide variety of molecules ranging from small molecules to large proteins, as well as a variety of interaction pairs. For example, representative ligands include, but are not limited to, the agents listed below (representative analyte interaction partners are parenthetically identified): antigen (specific antibody), antibody (antigen), hormone (hormone receptor), hormone receptor (hormone), polynucleotide (complementary polynucleotide), avidin/streptavidin (biotin), biotin (avidin/streptavidin), enzyme (enzyme substrate or inhibitor), enzyme substrate or inhibitor (enzyme), lectins (specific carboxyhydrate), specific carboxyhydrate (lectins), lipids (lipid binding proteins or membrane-associated proteins), lipid binding proteins or membrane-associated proteins (lipids), polynucleotides (polynucleotide binding proteins), polynucleotide binding proteins (polynucleotides), receptor (transmitter), transmitter (receptor), drug (target), target (drug), as well as more general types of interactions such as protein (protein), protein (polynucleotide), polynucleotide (protein), DNA (DNA), DNA (RNA), and RNA (DNA) interactions.

The biosensors of this invention are used in conjunction with a detection device which may employ a variety of detection methods. Typically, such methods include, but are not limited to, mass detection methods, such as piezoelectric, optical, thermo-optical and surface acoustic wave (SAW) device methods, and electrochemical methods, such as potentiometric, conductometric, amperometric and capacitance methods. With regard to optical detection methods, representative methods include those that detect mass surface concentration, such as reflection-optical methods, including both internal and external reflection methods,

angle, wavelength or phase resolved, for example ellipsometry and evanescent wave spectroscopy (EWS), the latter including surface plasmon resonance (SPR) spectroscopy, Brewster angle refractometry, critical angle refractometry, frustrated total reflection (FTR), evanescent wave ellipsometry, scattered total internal reflection (STIR), optical wave guide sensors, evanescent wave-based imaging, such as critical angle resolved imaging, Brewster angle resolved imaging, SPR angle resolved imaging, and the like. Further, photometric methods based on, for example, evanescent fluorescence (TIRF) and phosphorescence may also be employed, as well as waveguide interferometers. While the present invention is hereinafter illustrated in the context of SPR spectroscopy, it is to be understood that the invention is not limited in this manner.

Moreover, it is to be understood that the term "biosensor," as used within the context of the present invention, is to be construed broadly so as to encompass any analytical device capable of detecting biomolecular interactions between one or more analytes and one or more sensing surface-bound ligands, provided that the device includes at least one sensing element coupled to a transducer. Accordingly, the term "biosensor" covers not only analytical devices that use flow systems to contact sample with one or more sensing surfaces like the microfluidic structures of the BIAcore instrument, but also covers analytical devices that use non-flow systems to contact sample with one or more sensing surfaces like the cuvette structure employed by some biosensor instruments. As such, the present invention is applicable to both types of flow and non-flow systems.

As mentioned above, the BIAcore instrument (Biacore AB, Uppsala, Sweden) is an SPR-based, affinity biosensor system which is particularly useful for determining affinity and kinetics of interactions between two or more biomolecules (Karlsson et al., *Structures of Antigens*, M. H. V. Van Regenmortal (Editor), vol. 1., chap. 7, pp. 127-148, CRC Press, 1992; Karlsson et al., *J. Immunol. Meth. 200*:121-133, 1997; Myszka, *Current Opinion in Biotechnology 8*:50-57, 1997), receptor-ligand interactions (Cunningham et al., *J. Mol. Biol. 234*:554-563, 1993), and hybridizations (Persson et al., *Anal. Biochem. 246*:34-44, 1997). The BIAcore instrument is also used for quantitative analysis using antibodies as a specific reagent (Sternesjö et al., *Analytical Biochemistry 226*:175-81, 1995). The vast majority of such methods require regeneration of the biosensor surface before a measurement can be repeated. Thus, optimization of regeneration conditions is of both general and specific interest.

In the BIAcore instrument, the biosensor surface is preferably regenerated with a regeneration agent that does not influence the bound ligand in any permanent sense. Otherwise the system characteristics will change from measurement to measurement, which may result in data that is not truly comparable. Specifically, for kinetic assays, the model parameters may have different true values for each measurement, whereas for quantitative assays the calibration may become less valid because of a baseline shift during consequent measurements.

The present invention overcomes these problems, and provides further related advantages, by employing a small library of "stock solutions" that simplify identification and optimization of regeneration conditions for biosensor surfaces. The stock solutions of the present invention constitute different chemical properties which, either alone or in some combination, are representative of the vast majority of the regeneration agents presently employed for surface regeneration. Of these chemical properties, acid, base, ion strength, detergent, organic and chelating agents have been found to be sufficient in the context of this invention. Thus, the library of the present invention is made in the form of six stock solutions representing each of these properties. Each stock solution typically contains several compounds with similar main properties, but with differences in molecular structure, pKa, etc. In one embodiment, the regeneration effects of predefined mixes of the six stock solutions and water in various combinations are initially tested. In another embodiment, the regeneration effects of mixes of the six stock solutions with or without added water in various combinations are initially tested. Such mixtures of stock solutions, either with a single stock solution and water, or with at least two different stock solutions with or without added water, are referred to herein as "regeneration cocktails."

In order to determine the regenerative effects for each of the stock solutions that comprise the corresponding regeneration cocktails, a structured experimental design has been developed. More specifically, a multivariate approach or protocol is employed for identifying and optimizing the regeneration conditions for a particular biosensor surface. Such a structured experimental design forces the parameters under investigation, namely the six stock solutions, to vary in an uncorrelated manner. As a result, it is possible to estimate the degree of importance of each parameter. Moreover, a structured experimental design also allows conclusions to be drawn that are typically more reliable than from experiments where the parameters are allowed to vary only one at a time (Haaland, P.D. *Experimental Design in Biotechnology*; Marcel Dekker: New York, 1989; Box; Hunter; Hunter *Statistics for Experimenters*; John Wiley & Sons, 1978). As such, the experiments of

the present invention are designed with focus on ease-of-use rather than on getting maximal information from a given number of experiments. Thus, the surface-regeneration approaches of this invention results in experimental data that can be reliably evaluated without the need for any sophisticated mathematical or statistical analysis.

More specifically, in the practice of the present invention, a biosensor surface is sequentially contacted with each of a plurality of first regeneration cocktails made from the library of six stock solutions. As used herein, a "plurality" of first regeneration cocktails means at least two, typically at least eight, and preferably at least 18. Such first regeneration cocktails are aqueous solutions comprising at least one acidic, basic, ionic, organic, detergent, or chelating stock solutions. Further, in the practice of the present invention at least one of the first regeneration cocktails comprises a mixture of at least two different stock solutions selected from the above list. For purposes of convenience, the stock solutions of the present invention are abbreviated as follows: A (acidic), B (basic), I (ionic), O (organic), D (detergent) and C (chelating).

In addition, it should also be understood that each of the first regeneration cocktails comprise an aqueous mixture of water ("w") and at least one, and typically one or two, and possibly three or more stock solutions of the present invention. Representative first regeneration cocktails of this invention that contain one or two stock solutions are listed in Table 1, while representative first regeneration cocktails that contain three stock solutions are listed in Table 2, wherein each component is represented as an equal volume component. For example, the first regeneration cocktail "Aww" represents one volume of stock solution A mixed with two volumes of water, whereas "BAw" represents a mixture of one volume of stock solution B, one volume of stock solution A, and one volume of water. However, other ratios of water may also be used. To this end, water may be present in each of the first regeneration cocktails in an amount ranging from 0% to 95% by volume, typically from 20% to 50%, and preferably from 33% to 50%. (Note that first regeneration cocktails corresponding to "BIw" and "BOw" are not depicted as these mixtures may form precipitates, while the first regeneration cocktail corresponding to "IOw" is not depicted as this mixture may be too aggressive.)

Table 1

Representative First Regeneration Cocktails

Containing One Or Two Stock Solutions

Aww	Bww	Iww	Oww	Dww	Cww
BAw	BDw	BCw	AIw	AOw	ADw
ACw	IDw	ICw	DOw	DCw	OCw

Table 2

Representative First Regeneration Cocktails

Containing Three Stock Solutions

ABIw	ABDw	ABCw	AIDw	AICw	ADOw
ADCw	AOCw	BDOw	BOCw	IDCw	DOCw

As stated above, the stock solutions of the present invention are aqueous solutions comprising at least one acidic, basic, ionic, organic, detergent or chelating component. Typically, each stock solution contains a combination of two or more components of like kind (with the exception of the chelating stock solution as discussed below). The concentration of each component within each stock solution is typically high, in some cases approaching saturation. Employing high concentration stock solutions is preferable since it permits a wide range of concentrations following dilution with other stock solutions and/or water. Representative components for each stock solution of the chemical library are discussed further below.

Acidic stock solutions of the present invention comprise one or more acids having dispersed pKs ranging from about 2 up to about 7, and having a pH ranging from 1 up to about 7. Suitable acids in this context include both organic and inorganic acids such as arsenic acid, arsenious acid, o-boric acid, carbonic acid, chromic acid, germanic acid, hyrocyanic acid, hydrofluoric acid, hydrogen sulfide, hydrogen peroide, hypobromus acid, hypochlorous acid, hypoiodous acid, iodic acid, nitrous acid, periodic acid, o-phosphoric acid, phosphorous acid, pyrophosphoric acid, selenic acid, selenious acid, m-silic acid, o-silic acid, sulfuric acid, sulfurous acid, telluric acid, tellurous acid, tetraboric acid, acetic acid, acetoacetic acid, acrylic acid, adipamic acid, adipic acid, d-alanine, allantoin, alloxanic acid, glycine, o-aminobenzoic acid, m-aminobenzoic acid, p-aminobenzoic acid,

o-aminobenzsulfonic acid, m-aminobenzsulfonic acid, p-aminobenzsulfonic acid, ainsic acid, o-beta-anisylpropionic acid, m-beta-anisylpropionic acid, p-beta-anisylpropionic acid, ascorbic acid, DL-aspartic acid, barbituric acid, benzoic acid, benzosoulfonic acid, bromoacetic acid, o-bromobenzoic acid, m-bromobenzoic acid, n-butyric acid, iso-butyric acid, cyclopropane-1:1-dicarboxylic acid, DL-cystein, L-cystein, dichloroacetic acid, dichloroacetylacetic acid, 2,3-dichlorophenol, 2,2-dihydroxybenzoic, 2,5-dihydroxybenzoic, 3,4-dihydroxybenzoic, 3,5-dihydroxybenzoic, dihydroxymalic acid, dihydroxytartaric acid, dimethylglycine, dimethylmalic acid, dimethylmalonic acid, dinicotinic acid, 2,4-dinitrophenol, 3,6-dinitrophenol, diphenylacetic acid, ethylbenzoic acid, ethylphenylacetic acid, fluorobenzoic acid, formic acid, fumaric acid, furancarboxylic acid, furoic acid, cacodylic acid, n-caproic acid, iso-caproic acid, chloroacetic acid, o-chlorobenzoic acid, m-chlorobenzoic acid, p-chlorobenzoic acid, o-chlorobutyric acid, m-chlorobutyric acid, p-chlorobutyric acid, o-chloroinnamic acid ,m-chloroinnamic acid, p-chloroinnamic acid, o-chlorophenoxyacetic acid, m-chlorophenoxyacetic acid, p-chlorophenoxyacetic acid, o-chlorophenylacetic acid, m-chlorophenylacetic acid, p-chlorophenylacetic acid, beta-(o-chlorophenyl) propionic acid, beta-(m-chlorophenyl) propionic acid, beta-(p-chlorophenyl) propionic acid, alfa-chloropropinic acid, beta-chloropropionic acid, cis-cinnamic acid, trans-cinnamic acid, citric acid, o-cresol, m-cresol, p-cresol, trans-crotonic acid, cyanoacetic acid, gamma-cyanobutyric acid, o-cyanophenoxyacetic acid, p-cyanophenoxyacetic acid, cyanopropionic acid, cycloheaxane-1:1-dicarboxylic acid, gallic acid, glutamaric acid, glutaric acid, glycerol, glycine, glycol, glycolic acid, heptanoic acid, hexahydrobenzoic acid, hexanoic acid, hippuric acid, histidine, hydroquinone, o-hydroxybenzoic acid, m-hydroxybenzoic acid, p-hydroxybenzoic acid, beta-hydroxybutyric acid, gamma-hydroxybutyric acid, beta-hydroxypropionic acid, gamma-hydroxyquinoline, iodoacetic acid, o-iodobenzoic acid, m-iodobenzoic acid, itaconic acid, lactic acid, lutidinic acid, lysine, maleic acid, malic acid, malonic acid, DL-mandelic acid, mesaconic acid, mesitylenic acid, methyl-o-aminobenzoic acid, methyl-m-aminobenzoic acid, methyl-p-aminobenzoic acid, o-methylcinnamic acid, m-methylcinnamic acid, p-methylcinnamic acid, beta-methylglutaric acid, n-methylglycine, methylmalonic acid, methylsuccinic acid, o-monochlorophenol, m-monochlorophenol, p-monochlorophenol, o-phthalic acid, m-phthalic acid, p-phthalic acid, picric acid, pimelic acid, propionic acid, iso-propylbenzoic acid, 2-pyridinecarboxylic acid, 3-pyridinecarboxylic acid, 4-pyridinecarboxylic acid, pyrocatcchol, quinolinic acid, Resorcinol, Saccarin, suberic acid, succinic acid,

sulfanilic acid, naphtalenesulfonic acid, alfa-naphthoic acid, beta-naphthoic acid, alfa-napthol, beta-napthol, nitrobenzene, o-nitrobenzoic acid, m-nitrobenzoic acid, p-nitrobenzoic acid, o-nitrophenol, m-nitrophenol, p-nitrophenol, o-nitrophenylacetic acid, m-nitrophenylacetic acid, p-nitrophenylacetic acid, o-beta nitrophenylpropionic acid, p-beta nitrophenylpropionic acid, nonanic acid, octanic acid, oxalic acid, phenol, phenylacetic acid, o-phenylbenzoic acid, gamma-phenylbutyric acid, alfa-phenylpropionic acid, beta-phenylpropionic acid, alfa-tataric acid, alfa-tartaric acid, meso-tartaric acid, theobromine, terephtalic acid, thioacetic acid, thiophenecarboxylic acid, o-toluic acid, m-toluic acid, p-toluic acid, trichloroacetic acid, trichlorophenol, 2,4,6-trihydroxybenzoic acid, trimethylacetic acid, 2,4,6-trinitrophenol, tryptophan, tyrosine, uric acid, n-valeric acid, iso-valeric acid, veronal, vinylacetic acid, and xanthine. In one embodiment, the acidic stock solution is a mixture of acids comprising oxalic (0.15M), phosphoric (0.15M), formic (0.15M), and malonic acid (0.15M), wherein the mixture contains nearly equal volume portions of each acid adjusted to about pH 5.0 with NaOH.

Basic stock solutions of the present invention comprise one or more bases having dispersed pKs ranging from in excess of 7 up to about 12, and a pH ranging from 7 to 12. Suitable bases include acetamide, acridin, alfa-alanin, gylcyl alanin, methoxy (DL) - alanin, phenyl alanin, allothreonin, n-amylamine, aniline, n-allyl aniline, 4-(p-aminobenzoyl) aniline, 4-benzyl aniline, 2-bromo aniline, 3-bromo aniline, 4-bromo aniline, 4-bromo-N,N,dimethyl aniline, o-chloro aniline, m- chloro aniline, p- chloro aniline, 3-bromo-N,N,dimethyl aniline, 4-bromo-N,N,dimethyl aniline, 3,5-dibromo Aniline, 2,4-dichloro aniline, N,N-diethyl aniline, N,N-dimethyl-3-nitro aniline, N-ethyl aniline, 2-fluoro aniline, 3- fluoro aniline, 4- fluoro aniline, 2-iodo aniline, N-methyl aniline, N-methylthio aniline, 3- nitro aniline, 4- nitro aniline, 2-sulfonic acid aniline, 3-sulfonic acid aniline, 4-sulfonic acid aniline, brucine, 1-amino-3-methyl-butane, 2-amino-4-methyl-butane, 1,4-diamino-butane, n-butylamine, t-butylamine, 4-amino butyric acid, lycyl-2-amino n-butyric acid, cacodylic acid, beta-chlortriethylammonium, cinnoline, codeine, n-butyl-cyclohexanamin, cyclohexanamin, cystin, n-decylamine, diethylamine, diisobutylamine, diisopropylamine, dimethylamine, n-diphenylamine, n-dodecaneamine, d-ephedrine, l-ephedrine, 1-amino-3-metoxy-ethane, 2-amino ethanole, o-anisidine, m-anisidine, p-anisidine, arginin, asparagin, glycylasparagin, DL-aspartic acid, azetidin, aziridine, 4-aminoazo benzene, 2-aminoethyl benzene, 4-dimethylaminoazo benzene, benzidine, benzimidazole, 2-ethyl benzimidazole, 2-methyl benzimidazole, 2-phenyl

benzimidazole, 2-amino benzoic acid, 4-amino benzoic acid, benzylamine, betaine, 2-amino biphenyl, trans-bornylamine, ehtylamine, ethylenediamine, l-Glutamic acid, alfa-monoethyl glutamic acid, l-glutamine, l-glutathione, glycine, n-acetyl glycine, dimethyl glycine, glycyl glycine, glycylglycyl glycine, leucyl glycine, methyl glycine, phenyl glycine, N,n-propyl glycine, tetraglycyl glycine, glycylserine, hexadecanamine, 1-amino heptan, 2-amino-heptan, 2-metylamino heptan, hexadecanamine, hexamethylenediamine, 6-amino hexanoic acid, n-hexylamine, dl-histidine, beta-analyl histidine, imidazol, 2,4-dimethyl imidazol, 1-methyl imidazol, 1-amino indane, 2-amino isobutyric acid, isoleucin, isoquinolin, 1-amino isoquinolin, 7-hydroxy isoquinolin, L-leucin, glycyl leucin, methionin, metylamine, morphine, morpholine, 1-amino-6-hydroxy naphtalene, dimethylamino naphtalene, alfa-naphthylamine, beta-napthylamine, piperazine, 2,5,dimetyl(trans)piperazine, piperidine, 3-acetyl piperidine, 1-n-butyl piperidine, 1,2-dimethyl piperidine, 1-ethyl piperidine, 1-methyl piperidine, 2,2,6,6,tetramethyl piperidine, 2,2,4-trimethyl piperidine, proline, hydroxyproline, 1-amino-2,2-dimethylpropane, 1,2-diaminopropane, 1,3-diaminopropane, 1,2,3-triaminopropane, 3-amino propanoic acid, propylamine, pteridine, 2-amino-4-hydroxy pteridine, 2-amino-4,6-dihydroxy pteridine, 6-chloro pteridine, 6-hydroxy-4-methyl pteridine, purine, n-methyl alfa-naphtylamine, cis-neobornylamine, nicotine, n-nonylamine, norleucine, octadecanamine, octylamine, ornithine, papaverine, 3-amino pentane, 3-amino-3-methyl pentane, n-pentadecylamine, 5-amino pentanoic acid, perimidine, phenanthridine, 1,10-phenanthroline, o-phenetidine, m-phenetidine, p-phenetidine, alfa-picoline, beta-picoline, gamma-picoline, pilocarpine, 6-amino purin, 2-dimethylaminopurine, 8-hydroxy purin, pyrazin, 2-methylpyrazine, methylaminopyrazine, pyrdazine, 2-aminopyrimidine, 2-amino-4,6-dimethylpyrimidine, 2-amino-5-nitro pyrimidine, pyridine, 2-amino pyridine, 4-amino pyridine, 2-benzyl pyridine, 3-bromo pyridine, 3-chloro pyridine, 2,5-diamino pyridine, 2,3-dimethyl pyridine, 2,4-dimethyl pyridine, 2-ethyl pyridine, 2-formyl pyridine, a-hydroxy pyridine, 4-hydroxy pyridine, methoxy pyridine, 4-methylamino pyridine, 2,4,6,trimetyl pyridine, pyrrolidine, 1,2-dimethyl pyrrolidine, n-methyl pyrrolidine, quinazoline, 5-hydroxy quinazoline, quinine, quinoline, 3-amino quinoline, 3-bromo quinoline, 8-carboxy quinoline, 3-hydroxy quinoline, 8-hydroxy quinoline, 8-hydroxy-5-sulfo quinoline, 6-methoxy quinoline, 2-methyl quinoline, 4-methyl quinoline, 5-methyl quinoline, quinoxaline, serine, strychnine, taurine; tetradecaneamin, thiazole, 2-aminothiazole, threonine, o-toluidine, m-toluidine, p-toluidine, 2,4,6-triamino-1,3,5-triazine, tridecanamine,

triethylamine, trimethylamine, tryptophan, tyrosine, urea, valine, ammonium hydroxide, arsenous oxide, beryllium hydroxide, calcium hydroxide, deuteroammonium hydroxide, hydrazine, hydroxylamine, lead hydroxide, silver hydroxide, and zinc hydroxide. In one embodiment, the basic stock solution is a mixture of bases comprising ethanolamine (0.20M), sodium phosphate (0.20M), piperazin (0.20M), and glycine (0.20M), wherein the mixture contains nearly equal volume portions of each base adjusted to about pH 9.0.

Ionic stock solutions of the present invention comprise a mixture of at least two ionic compounds. Suitable ions of the at least two ionic compounds include PO_4^{3-}, SO_4^{2-}, CH_3COO^-, Br^-, NO_3^-, NO_2^-, ClO_4^-, Cl^-, F^-, I^-, CF_3COO^-, SCN^-, Cl_3COO^-, CCl_3COO^-, $(CH_3)_4N^+$, NH_4^+, Rb^+, K^+, Na^+, Cs^+, Li^+, Mg^{2+}, Ca^{2+}, Sr^{2+}, and Ba^{2+}. Such ions may be generated by adding water to a suitable precursor or salt, such as potassium thiocyanate, magnesium chloride, urea, guanidine HCl, sodium chloride, potassium chloride, ammonium chloride, or lithium chloride. In one embodiment, the ionic stock solution comprises a mixture of potassium thiocyanate (0.46M), magnesium chloride (1.83M), urea (0.92M), and guanidine HCl (1.83M).

Organic stock solutions of the present invention comprise a mixture of at least two organic solvents. Suitable organic solvents include DMSO, formamide, ethanol, acetonitrile, 1-butanol, acetone, methyl acetate, dichloroethane, chloroform, methyl alcohol, tetrahydrofuran, *n*-hexane, diisopropyl ether, ethyl acetate, ethyl alcohol, butanone, n-hexane, 2-propanol, 1,2-dichloroethane, flourobenzene, acetone, trichloroethylene, triethylamine, 1-propanol, butyronitrile, 2-butanol, nitromethane, dioxane, 2,2-dimethylpropanole, 3-pentanone, piperazine, 3-propanol, pyridin, 1-butanol, acetic acid, 2-metoxy ethanol, 3-methyl-1-butanol, chlorobenzene, acetic anhydride, dimethylformamide, methoxybenzene, methylbutylketone, bromobenzene, 1-hexanol, n-methyl formamide, aniline, iodobenzene, glycol, phenyl acetate, n-methyl formamide, benzyl alcohol, formamide, nitrophenol, diethyleneglycol, diphenylether, sulfolan, diethylether, methylene chloride, carbon disulfide, carbon tetrachloride, benzene, acetonitrile, toluene, dibutyl ether, mesitylen (*e.g.*, dimethylbenzene), ortho-dichlorobenzene, and benzonitrile. In one embodiment, the organic stock solution comprises nearly equal volume portions of DMSO, formamide, ethanol, acetonitrile, and 1-butanol.

Detergent stock solutions of the present invention comprise a mixture of at least two detergent agents such as anionic detergents, cationic detergents, zwitterionic detergents, and nonionic detergents. In one embodiment, the detergent stock solution comprises CHAPS (0.3% (w/w)), Zwittergent 3-12 (0.3% (w/w)),

Tween 80 (0.3% (v/v)), Tween 20 (0.3% (v/v)), and Triton X-100 (0.3% (v/v)) (commercially available from Sigma).

Chelating stock solutions of the present invention comprise at least one chelating agent including EDTA, EGTA, NTA, DCYTA, GLEDTA, ETHEDTA, IDA, Kryptand 222B, Kryptofix® 221, Kryptofix® 222, and crown ethers (commercially available from Fluka). In one embodiment, the chelating stock solution comprises a 20mM EDTA solution.

For purposes of illustration, components for each of six representative stock solutions of the present invention are disclosed in Table 3.

Table 3

Representative Stock Solutions

Acidic	Basic	Ionic	Organic	Detergent	Chelating
oxalic	ethanolamine	KSCN	DMSO	CHAPS	EDTA
phosphoric	Na3PO4	MgCl2	formamide	Zwittergent	
formic	piperazine	urea	ethanol	tween 80	
malonic	glycine	guanidine	acetonitrile	tween 20	
			1-butanol	triton X-100	

In the practice of the present invention a biosensor surface (having a surface-bound ligand and an analyte associated therewith) is sequentially contacted with each of a plurality of first regeneration cocktails, wherein each of the first regeneration cocktails is an aqueous solution comprising at least one acidic, basic, ionic, organic, detergent or chelating stock solution as disclosed above, and wherein at least one of the first regeneration cocktails comprises a mixture of at least two of the stock solutions. For example, in the context of the BIAcore instrument, a plurality of first regeneration cocktails comprising various combinations of the stock solutions and/or water are sequentially introduced into the running buffer so as to come into contact with the biosensor surface to be regenerated.

In one embodiment, each regeneration cocktail is introduced into the running buffer of the BIAcore instrument (*e.g.*, in random order) by a controlled injection of 30 second duration (flow 20µl/min) into an associated portal. Upon contacting the biosensor surface with each of the plurality of first regeneration cocktails, the regeneration effect for each of the cocktails is measured as the

percentage loss of analyte due to the cocktail injection. As used herein, the "regeneration effect" (R_e) is calculated by the following Equation (1):

$$R_e = \{(\text{Analyte loss})/(\text{Analyte level})\} \times 100 \qquad (1)$$

Based on these measurements, the first regeneration cocktails having the highest regeneration effect are identified. For example, the R_e values for each of the plurality of first regeneration cocktails may be plotted on a bar diagram (*e.g.*, R_e values along the ordinate, first regeneration cocktails along the abscissa) so as to facilitate visual evaluation of the regeneration effect. In addition, during the step of sequentially contacting the biosensor surface with each of the plurality of first regeneration cocktails as set forth above (or with subsequent regeneration cocktails as set forth below), the biosensor surface may be contacted with an additional quantity of analyte to associate additional analyte with the surface-bound ligand. In particular, when the analyte level has decreased to about one third of its maximum level, the biosensor surface may be contacted with an additional quantity of analyte. It should also be understood that the analyte eluted from the biosensor surface may be collected for subsequent analysis (*e.g.*, mass spectroscopy analysis).

In a further embodiment, the first regeneration cocktails having the two or three (or more) highest R_e values are identified (*e.g.*, from the aforementioned group of 18 first regeneration or screening cocktails identified in Table 1), and the two or three stock solutions that comprise these first regeneration cocktails are selected for further investigation and/or optimization.

Mixtures of these selected stock solutions, either with water or with other selected stock solutions, are referred to herein as "second regeneration cocktails." In order to generate a plurality of second regeneration cocktails, two different experimental designs may be employed to aid in determining possible two or three stock solution combinations for further investigation. As illustrated in Figure 2, a two-dimensional mixture optimization design ("MO2D") may be employed when two stock solutions have been selected; whereas, as illustrated in Figure 3, a three-dimensional mixture optimization design ("MO3D") may be employed when three stock solutions have been selected.

Referring to Figure 2, exemplary compositions for each of the two-stock second regeneration cocktails are denoted both in the table and on the design diagram. Specifically, the MO2D experimental design may, for example, result in 10 second regeneration cocktails of varying compositions. In addition, for

those second regeneration cocktails in which the sum of volume percent of the two stock solutions is lower than 100%, water is added as a third solution. As exemplified, the calculated R_e values for each of the 10 second regeneration cocktails may be plotted in the corresponding positions of the design diagram of Figure 2, thereby facilitating visual interpretation of the experimental results.

Similarly, referring to Figure 3, the composition of each of the three-stock second regeneration cocktails are denoted on the design diagram. Specifically, the MO3D experimental design results, for example, in seven second regeneration cocktails of varying compositions. As exemplified, the calculated R_e values for each of the seven second regeneration cocktails may be plotted in the corresponding positions of the design diagram of Figure 3, thereby facilitating visual interpretation of the experimental results.

It should be understood, however, that the compositions of stock solutions and/or water as depicted in Figures 2 and 3 are exemplary of a representative embodiment of the present invention. Accordingly, compositions of stock solutions in ratios other than those depicted are possible for the second regeneration cocktails. For example, the second regeneration cocktails may comprise four (or more) different stock solutions.

In another aspect of the present invention, a plurality of second regeneration cocktails (such as those having compositions corresponding to either the MO2D or MO3D experimental designs discussed above) are sequentially contacted with the biosensor surface. As with the first regeneration cocktails, upon contacting the biosensor surface with each of the plurality of second regeneration cocktails, the regeneration effect for each of the second regeneration cocktails is measured as the percentage loss of analyte due to the cocktail injection. Based on these measurements, the second regeneration cocktails having the highest regeneration effect are identified. In general, the second regeneration cocktail with the highest R_e value is sufficient for selection as the optimized regeneration solution.

Accordingly, in one embodiment, the method of this invention comprises the following steps:

(a) sequentially contacting the biosensor surface with each of a plurality of first regeneration cocktails, wherein each of said first regeneration cocktails is an aqueous solution comprising at least one acidic, basic, ionic, organic, detergent or chelating stock solution, and wherein at least one of said first regeneration cocktails comprises a mixture of at least two of said stock solutions;

(b) measuring the regeneration effect for each of said plurality of first regeneration cocktails to determine which of said plurality of first regeneration cocktails have the highest measured regeneration effect;

(c) selecting at least two different stock solutions present in said plurality of first regeneration cocktails having the highest measured regeneration effect;

(d) combining said at least two different stock solutions in various ratios to generate a plurality of second regeneration cocktails;

(e) sequentially contacting the biosenor surface with each of said plurality of second regeneration cocktails; and

(f) determining the regeneration effect of each of said plurality of second regeneration cocktails and therefrom identifying a second regeneration cocktail as the optimized regeneration solution.

However, in those instances where the optimized regeneration solution has an unsatisfactory R_e value (*e.g.*, when the R_e value is less than 90), additional iterations with a further (*e.g.*, third) regeneration cocktails may be performed - that is, the ratio of stock solutions that comprise the second regeneration cocktails may be varied so as to generate a plurality of third regeneration cocktails. The third regeneration cocktails are then sequentially contacted with the biosensor surface and the regeneration effect for each of the cocktails is measured as before. In general, the third regeneration cocktail with the highest R_e value may be selected as the optimized regeneration solution. The above steps may be repeated until the optimized regeneration solution is identified.

Thus, the method of this invention further comprises, after step (f) above, the following additional steps:

(g) combining said at least two different stock solutions in different ratios than step (d) to generate a plurality of third regeneration cocktails;

(h) sequentially contacting the biosensor surface with each of said plurality of third regeneration cocktails; and

(i) determining the regeneration effect of each of said plurality of third regeneration cocktails and therefrom identifying a third regeneration cocktail as the optimized regeneration solution. The above steps may be repeated until the optimized regeneration solution is identified.

In another embodiment, the present invention is directed to a reagent kit comprising at least two different stock solutions selected from the library of six stock solutions; namely, a basic stock solution, an ionic stock solution, an organic

stock solution, a detergent stock solution, and a chelating stock solution. The reagent kit may comprise at least three different stock solutions, four to five stock solutions, or six different stock solutions.

In yet another embodiment, the present invention is directed to a computer system (operatively connected to a device) for selecting an optimized regeneration solution for the regeneration of a biosensor surface having a surface-bound ligand and an analyte associated with the ligand. Specifically, the computer system has associated computer software and/or hardware capable of performing the following steps:

(a) instructing a device to combine a series of stock solutions in various ratios to generate a plurality of first regeneration cocktails;

(b) sequentially controlling the device to contact the biosensor surface with each of said plurality of first regeneration cocktails;

(c) determining the regeneration effect of each of said first regeneration cocktails on the biosensor surface based on measurements received from the device;

(d) selecting at least two different stock solutions having the highest regeneration effect;

(e) instructing the device to combine a subset of said at least two different stock solutions in varying ratios to generate a plurality of second regeneration cocktails;

(f) sequentially controlling the device to contact the biosenor surface with each of said second regeneration cocktails; and

(g) determining the regeneration effect of each of said second regeneration cocktails based on measurements received from the device, and therefrom identifying a second regeneration cocktail as the optimized regeneration solution.

In a further aspect of this embodiment, the method further comprises, after step (g), the following additional steps:

(h) instructing the device to combine the subset of said at least two different stock solutions in different ratios than step (e) to generate a plurality of third regeneration cocktails;

(i) sequentially controlling the device to contact the biosensor surface with each of said third regeneration cocktails; and

(j) determining the regeneration effect of each of said third regeneration cocktails based on measurements received from the device, and

therefrom identifying a third regeneration cocktail as the optimized regeneration solution.

In yet another aspect of this embodiment, the computer system substantially automates the routine illustrated in Figures 4A and 4B. Specifically, Figures 4A and 4B provide a flowchart illustrating the steps for identifying and optimizing the regeneration conditions for a biosensor surface of, for example, a BIAcore instrument. Referring to Figure 4A, an appropriate ligand (*i.e.*, selected from a ligand and analyte interaction pair under investigation) is immobilized onto the biosensor surface in step 301. In general, there should be enough immobilized ligand so as to obtain 300-1000 RU maximal binding capacity. Following the ligand immobilization, a plurality of first regeneration cocktails are made (not shown) from the previously disclosed library of six stock solutions, wherein at least one of the first regeneration cocktails comprises a mixture of at least two different stock solutions. While maintaining the running buffer, the baseline of the system is noted in step 302. Next, analyte is injected into the running buffer for a selected period of time (step 303) in order to associate the analyte with the immobilized ligand. One of the previously prepared regeneration cocktails is selected (*e.g.*, randomly) and injected over a 30 second period into the running buffer in step 304. The regeneration effect (R_e) value for the selected regeneration cocktail is then calculated in step 305.

The regeneration-optimization protocol then decides whether there are any other regeneration cocktails available for further analysis (step 306). If there are additional regeneration cocktails available, the regeneration-optimization protocol determines whether there is a sufficient about of analyte still associated with the immobilized ligand (step 307). In general, when the analyte level has decreased to about one third of its maximum level, the biosensor surface should be contacted with additional analyte. Accordingly, if there is not enough analyte, additional analyte is injected into the running buffer in step 308. On the other hand, if there is enough analyte associated with the immobilized ligand, then one of the remaining regeneration cocktails is selected (*e.g.*, randomly) and injected into the running buffer as before (step 309). The regeneration effect (R_e) value for the selected regeneration cocktail is then calculated in step 310. The regeneration-optimization protocol then decides whether there are still any other regeneration cocktails available for further analysis (*i.e.*, "loops-back" to step 306).

If there are not any additional regeneration cocktails available for further analysis (*see* Figure 4B), then the regeneration-optimization protocol determines whether any of the analyzed regeneration cocktails provides a satisfactory

regeneration of the biosensor surface (step 311). In general, a satisfactory regeneration of the biosensor surface is achieved when the regeneration effect (R_e) is greater than 99% for any of the analyzed regeneration cocktails. If the analyzed regeneration cocktails are not satisfactory, then the regeneration-optimization protocol selects the two or three stocks solutions (step 312) from the two or three first regeneration cocktails having the highest regeneration effect (R_e) values for further investigation and/or optimization (*i.e.*, for further formulation into second regeneration cocktails).

The regeneration-optimization protocol then determines whether three stock solutions have been selected for further investigation and/or optimization (step 313). If three stock solutions have not been selected, that necessarily means that only two stock solutions have been selected. Accordingly, in step 314 a plurality of second regeneration cocktails are prepared in accordance with the compositions of the previously disclosed two-dimensional mixture optimization design ("MO2D"). As before, one of these second regeneration cocktails is selected (*e.g.*, randomly) and injected over a 30 second period into the running buffer in step 304 (*i.e.*, "loops-back" to step 304), and the subsequent steps of the regeneration-optimization protocol are repeated. If, on the other hand, three stock solutions have been selected, then a plurality of second regeneration cocktails are prepared in step 315 in accordance with the compositions of the previously disclosed three-dimensional mixture optimization design ("MO3D"). Again, one of these second regeneration cocktails is selected (*e.g.*, randomly) and injected over a 30 second period into the running buffer in step 304 (*i.e.*, "loops-back" to step 304), and the subsequent steps of the regeneration-optimization protocol are repeated.

The above-identified regeneration-optimization protocol will usually result in regeneration cocktail that provides satisfactory regeneration of the biosensor surface (*i.e.*, regeneration effect (R_e) value that is greater than 99%). If, however, no satisfactory regeneration is achieved, then other combinations and/or different ratios of the stock solutions may be tested. In addition, the pH of the stock solutions may also be adjusted, and the cocktails may be injected into the running buffer in several short pulses as opposed to a constant flow. Moreover, for those regeneration cocktails that have substantially different compositions than those of the predefined mixing ratios as set forth above (*e.g.*, a combination of four stock solutions), or if the cocktail composition has significantly deviated from those previously tested, a check for precipitation should be performed by refrigerating the cocktail for at least one

hour. The precipitation check may avoid injections of particles that can clog the microfluidic cartridge of, for example, the BIAcore instrument.

In yet a further embodiment, the present invention is directed to a computer-readable medium containing the instructions for performing the above-identified steps for selecting an optimized regeneration solution for the regeneration of a biosensor surface having a surface-bound ligand and an analyte associated with the ligand.

The above disclosure has primarily focused on the selection of an optimized regeneration solution for regenerating a biosensor surface having a surface-bound ligand and analyte associated therewith. In a related embodiment of this invention, measuring the analyte-ligand binding interaction during association, steady state, and dissociation conditions (including regeneration) with the various above-identified stock solutions (including diluted forms thereof) is performed to provide information concerning the potential activity or functionality of the ligand and/or analyte. More specifically, by determining the kinetic parameters related with the association and dissociation of the analyte-ligand binding pair (as well as analogues thereof) in several different characterization solutions or running buffers of the present invention, structure-activity relationships (SARs) of the ligand and/or analyte may be predicted. Such information is referred to herein as a "characteristic" of the ligand and/or analyte, while the process for determining the same is referred to as "characterization." In addition, as used herein, the term "characterization solution" encompasses the above-identified stock solutions and regeneration cocktails made therefrom, as well as more dilute solutions thereof. As such, the term "characterization solution" is to be construed broadly so as to covers a full spectrum of possible solution concentrations (*e.g.*, 1pM aqueous solution to saturation).

As noted above, characterization of the ligand and/or analyte of the ligand-analyte pair has tremendous value, particularly in the context of predicting SAR. In the practice of this invention, this may be accomplished by contacting a biosensor surface (having a surface-bound ligand) with a plurality of characterization solutions of this invention and, while the characterization solution is in contact with the biosensor surface, introducing an analyte of interest into the characterization solution (*e.g.*, running buffer) for a selected period of time, measuring the biospecific interaction effect (*e.g.*, association rate, analyte surface concentration at steady state, dissociation rate and/or regeneration effect) for each of said plurality of characterization solutions, and therefrom characterizing the ligand and/or analyte. Such characterization may then be compared with similar characterizations made for

one or more test molecules, thereby allowing one to predict the SAR of the ligand and/or analyte of interest. For example, a mathematical model may be used that, given the analyte or ligand structure, predicts the analyte or ligand activity. In addition, the properties of the mathematical model may be used for characterization and communication of the biospecific interaction effect of the analyte or ligand.

Accordingly, in another aspect of this invention, a method for characterizing a ligand and/or analyte associated with a biosensor surface is disclosed, wherein the method comprises the following steps:

(a) sequentially contacting the biosensor surface having a surface-bound ligand with each of a plurality of characterization solutions;

(b) introducing the analyte into each of said plurality of characterization solutions so as to interact the analyte with the surface-bound ligand;

(c) measuring at least one of the association rate, surface-bound analyte concentration, dissociation rate, and regeneration effect of the analyte-ligand interaction for each of said plurality of characterization solutions; and

(d) characterizing the ligand and/or analyte associated with the biosensor surface based on at least one of the association rate, surface-bound analyte concentration, and dissociation rate of the analyte-ligand interaction for each of said plurality of characterization solutions.

In a further aspect of this embodiment, the method further comprises, after step (d), the following additional step:

(e) comparing the characterization of the ligand and/or analyte associated with the biosensor surface with a set of predetermined characterizations of other test molecules and thereby predicting the activity of the ligand and/or analyte associated with the biosensor surface.

It should be understood that, as used herein, the term "biospecific interaction effect" encompasses not only the aforementioned dissociation kinetic parameters, but also includes association kinetic parameters as well as the steady state aspects of the binding interaction between the ligand and analyte of interest (*e.g.*, affinity). Thus, as used in the context of this invention, biospecific interaction effect includes association rate, analyte surface concentration at steady state, dissociation rate, and regeneration effect, all of which may be measured with respect to each of the characterization solutions. The measured biospecific interaction effect may then be compared with a set of predetermined characterizations of other test molecules so as to provide valuable information concerning the potential activity or functionality of the ligand and/or analyte.

For example, a representative set of predetermined regeneration characterizations of test molecules (*i.e.*, ligand-analyte binding pairs) is illustrated in Figure 11, and further discussed in Example 1 below. Referring to Figure 11, a series of eight monoclonal antibodies and two polyclonal antibodies were each characterized against the 18 regeneration cocktails of Table 1 above. Assuming, for example, that the identify of one of the MAbs was not known, such as the MAb g2b_clone2, comparing the characteristics of that antibody with the characteristics of the remaining nine MAb, one can readily predict that the activity of g2b_clone2 would be most similar to that of g2a_clone3 and g2b_clone3. Accordingly, by characterizing a number of test molecules, SAR may be predicted by comparison of the sample molecule's characteristics with those of the predetermined test molecules.

Similarly, characterizations of analyte-ligand complexes may be performed by determining the kinetic (*i.e.*, association and dissociation) parameters of the interaction pair in a plurality of diverse buffers (*i.e.*, characterization solutions), preferably at two or more temperatures. The complete binding cycle (*i.e.*, both association and dissociation) for the analyte-ligand interaction pair is performed in each of the plurality of buffers, and kinetic constants may be calculated for each buffer. The plurality of buffers or characterization solutions may, for example, be selected from the above-identified stock solutions, the regeneration cocktails of Table 1 and/or Table 2, as well as diluted forms thereof. In addition, any one of these solutions may be varied by having other additives, including acids, bases, salts, organic solvents, and/or detergents. In general, for purposes of analysis, the plurality of diverse buffers may be selected according to the experimental design rules disclosed above.

In addition to these analyte-ligand characterizations, several analogues of the analyte and/or ligand may also be selected for characterization. That is, a plurality of analogues of the characterized analyte and/or ligand may be selected for subsequent characterizations, wherein the kinetic parameters of the analogue binding interactions are measured in each of the previously selected characterization solutions. For those analyte-ligand interaction pairs that have suitable analogues, a mathematical model may be employed to connect the measured kinetic constants with a structural description of the molecules of interest (*e.g.*, peptides, small molecules, proteins, and RNA/DNA), thereby allowing one to predict the SAR of other analogues. The properties of the mathematical model may be used to describe and communicate functional information in, for example, functional databases. Moreover, a collection of such characterizations of known analyte-ligand interaction

pairs and analogues thereof, has tremendous value in that they enable researchers to more accurately predict the activity and/or functionality of other closely related molecules. As used herein, the term "analogue" with respect to an analyte means a molecule capable of specifically binding to the ligand of the analyte-ligand interaction pair in the same or similar fashion as the analyte, whereas the term "analogue" with respect to a ligand means a molecule capable of specifically binding to the analyte of the analyte-ligand interaction pair in the same or similar fashion as the ligand.

Furthermore, the above-identified method for characterizing a ligand and/or analyte associated with a biosensor surface also finds utility in quantitative assays, and multi-buffer epitope mapping. For example, in developing quantitative assays for determination of vitamin concentration in food stuff, researchers are often interested in knowing how sensitive a specific reagent, typically a MAb, is to variations in its chemical environment. By using the characterization methods of this invention, researchers are able to choose the specific reagant that is least affected by changes in the chemical environment and by inhomogeneous buffers (*e.g.*, by monitoring how stable reagents for a quantitative assay behave with respect to inhomogeneous sample matrices).

Accordingly, a researcher may, for example, set up an inhibition assay for determination of biotin concentration in infant formulas, and prepare artificial samples to which a known quantity of biotin has been added. The artificial samples are preferably diverse (*e.g.*, having various salt concentrations, pH, fiber content, etc.). In this exemplary situation, the measured concentration of biotin in the artificial samples will deviate from the known concentration. By subsequently characterizing several different reagents (*e.g.*, at least three or four MAbs), a researcher may formulate a predictive model by determining biotin concentrations in several different infant formulas using all the reagents that were characterized. For example, those MAbs that give large deviations in the real samples may also give large deviations in the artificial samples, thereby allowing a predictive mathematical correlation to be expressed. Other substances that may be of interest for purposes of accurate concentration determinations include (but are not limited to) drug residues, hormones and toxins.

Similarly, researchers often desire to verify that manufactured proteins have their expected structures and are not point mutated. Accordingly, a researcher may, for example, immobilize on a biosensor surface three or four different MAbs directed to different epitopes on the same protein. The binding interactions between

the protein and three or four different MAbs may then be characterized by employing the characterization solutions and methodologies of this invention (*e.g.*, injecting the protein into several characterization solutions so as to interact with the surface-bound MAbs). In addition, similar protein analogues (*e.g.*, point mutated proteins) may further be characterized under the same conditions. A collection of such characterization data will serve as a unique "fingerprint" that reveal small protein changes, even if they are outside all MAb epitopes.

In still another aspect and related to the above discussed analyte-ligand interactions, this invention is also directed to a computer memory containing a data structure useful for communicating chemical perturbation information associated with an analyte-ligand interaction. More specifically, the data structure (*e.g.*, the functional database described above) comprises one or more kinetic parameters, where each kinetic parameter is expressed in terms of a mathematical model that describes the relation between the kinetic parameter (*e.g.*, association or dissociation rate constant) and the analyte-ligand interaction in a plurality of characterization solutions. In one embodiment, the plurality of characterization solutions corresponds to the stock solutions; however, such characterization solutions may cover the full spectrum of possible solution concentrations.

Because a plurality of characterization solutions are used to characterize the analyte-ligand interaction, the mathematical model derived therefrom may be used to predict the value of the kinetic parameter for the analyte-ligand interaction in other solutions. (Note that such various alternative chemical environments define a mental construct referred to herein as "chemical space.") In short, the data structure may be used to communicate chemical perturbation information associated with the analyte-ligand interaction. As used herein, the term "chemical perturbation" refers to changes in the surrounding chemical environment that define the chemical space. The communication of chemical perturbation information has great utility because a researcher, for example, may be able - in view of such information - control the affinity of a selected biomolecular interaction with a high degree of precision.

The aspect of this invention relating to a computer memory containing a data structure useful for communicating chemical perturbation information associated with an analyte-ligand interaction may be more specifically illustrated in the context of a high-level computer block diagram as is depicted in Figure 13. As shown, such a computer system for communicating chemical perturbation information 1300 contains one or more central processing units (CPUs) 1310,

input/output devices 1320, and the computer memory containing a data structure useful for communicating chemical perturbation information (memory) 1330. Among the input/output devices is a storage device 1321, such as a hard disk drive, and a computer-readable media drive 1322, which may be used to install software products, where the software products are provided on a computer-readable medium, such as a CD-ROM. The input/output devices also include a network connection 1323, through which the computer system 1300 may communicate with other connected computer systems, such as networks. The input/output devices may also contain a display 1324 and a data input device 1325.

The memory 1330 preferably contains an operating system 1331, such as MICROSOFT WINDOWS, for providing to other programs access to resources of the computer system. The memory 1330 preferably further contains software 1332. While the computer memory containing a data structure useful for communicating chemical perturbation information associated with an analyte-ligand interaction is preferably implemented on a computer system configured as described above, those skilled in the art will recognize that it may also be implemented on computer systems having different configurations.

In a related aspect, this invention is also directed to a generated data signal conveying a data structure useful for communicating chemical perturbation information associated with an analyte-ligand interaction. As above, the data structure comprises one or more kinetic parameters, where each kinetic parameter is expressed in terms of a mathematical model that describes the relation between the kinetic parameter and the analyte-ligand interaction in a plurality of characterization solutions, such that the data structure may be used to communicate chemical perturbation information associated with the analyte-ligand interaction.

For purposes of illustration and not limitation, the following examples more specifically discloses various aspects of the present invention.

EXAMPLE 1

This example illustrates a representative method of the present invention for identifying an optimized regeneration solution in connection with four different ligand-analyte (*i.e.*, antibody-antigen) systems.

MATERIALS AND METHODS

Stock Solutions

Six stock solutions, designated acidic (A), basic (B), ionic (I), organic (O), detergent (D) and chelating (C), were formed by combining the components listed in Table 4.

Table 4

Acidic	Basic	Ionic	Organic	Detergent	Chelating
oxalic	ethanolamine	KSCN	DMSO	CHAPS	EDTA
phosphoric	Na_3PO_4	$MgCl_2$	formamide	zwittergent	
formic	piperazine	urea	ethanol	tween 80	
malonic	glycine	guanidine	acetonitrile	tween 20	
			1-butanol	triton X-100	

TABLE LEGEND:

A: Equal volumes of oxalic acid, H_3PO_4, formic acid and malonic acid, each in 0.15M, mixed and adjusted to pH 5.0 with NaOH.

B: Equal volumes of ethanoleamine, Na_3PO_4, piperazin and glycine, each in 0.20M, mixed and adjusted to pH 9.0 with HCl.

I: A solution of KSCN (0.46M), $MgCl_2$ (1.83M), urea (0.92M), guanidinHCl (1.83M).

O: Equal volumes of DMSO, formamide, ethanol, acetonitrile and 1-butanol.

D: A solution of 0.3% (weight/weight) CHAPS, 0.3% (w/w) zwittergent 3-12, 0.3% (volume/volume) tween 80, 0.3% (v/v) tween 20 and 0.3% (v/v) triton X-100.

C: A 20mM EDTA solution.

First Regeneration Cocktails

Eighteen first regeneration cocktails were prepared as disclosed in Table 5. With regard to the first generation cocktails, equal volumes of the six stock solutions (designated A, B, I, O, D and C) and water were mixed in the proportions noted. For example, the first regeneration cocktail "Aww" was a mixture of one

volume stock solution A and two volumes water, whereas "BAw" contained one volume each of stock solution B, stock solution A and water.

Table 5

Aww	Bww	Iww	Oww	Dww	Cww
BAw	BDw	BCw	AIw	AUw	ADw
ACw	IDw	ICw	DOw	DCw	OCw

Ligands. Analytes and Coupling to Sensor Chip

Ligand polyclonal anti-GST Ab and analyte GST; ligands anti-p24 MAbs 609 and 576 and analyte p24; and ligand protein A and analytes MAbs g3_clone2, g2b_clone3, g2b_clone2, g2a_clone3, gla, g3-clone3, anti-theofyllin MAb 459, and anti-p24 MAb 609 were obtained from Biacore AB (Uppsala, Sweden). The protein A analytes anti-clenbuterol polyclonal Abs r200 and r154 were a gift from Dr. Chris Elliot (Belfast, Ireland).

CM5-chips, HBS running buffer and an Amine coupling kit (for use with the BIAcore instrument) were also obtained from Biacore AB. The ligands were coupled to CM-5 chips in BIACORE 1000 according to Jönsson et al (Jönsson et al., *BioTechniques 11*:620-27, 1991). The CM-5 surface was activated with a 7 minutes EDC/NHS pulse. The ligands, solved in 10mM acetate buffer pH 5.0, were then injected for 3-5 minutes. Next a 3.5 minutes pulse of 1.0 M ethanoleamine pH 8.5 deactivated the surface. The immobilization level for the ligands were typically about 4000 RU.

Run and Evaluation

BIACORE 1000 was programmed to test the regeneration effect of the 18 different first regeneration cocktails at two different cycles. First a 10 minute analyte injection (flow 2µl/min) was performed. The original baseline and the obtained analyte level were saved. Then, 30 second injections (flow 20µl/min) of the different cocktails were performed until the analyte level had decreased to 30% of the original value. When low analyte level was detected, a new analyte injection was performed, followed by more cocktail injections. The cocktails were injected in random order. BIACORE 1000 was equipped with a computer system having software with if-then capabilities which made possible a complete automation of the

experiments. However, the experiments could as well have been performed in manual mode as described below.

A regeneration effect value (R_e) was assigned for each of 18 first regeneration cocktails (*i.e.*, screening cocktails). This R_e value was calculated as the percentage loss of analyte due to the cocktail injection. Thus, with the initial baseline response level as reference, RU values of analyte loss and analyte level were calculated for each screening cocktail. In order to correct for dissociation of the ligand-analyte complex, the sensorgram before the screening cocktail injection was extrapolated to be comparable with the response level after the screening cocktail injection.

More specifically, Figure 5 shows a sensorgram from a regeneration-optimization protocol run with the MAb 609 - p24 system. Three analyte injections and seven cocktail injections are shown. The injections were performed in the following order: analyte p24, Cww, ADw, Bww, ICw, analyte p24, Aww, ODw, analyte p24 and AIw (numbered 1-10, respectively). For injection 3 (ADw) the extraction of analyte level (long arrow) and analyte loss (short arrow) are shown. The baseline decreased significantly during the first cocktail injections which is indicated by the extended original baseline. The peak in the beginning of injection 9 (analyte p24) was due to insufficient cleaning after injection 8 (ODw). Execution of an extraclean after injection 8 would provide sufficient cleaning of the microfluidic system.

Screening and Evaluation

The regeneration effect of the 18 different first regeneration cocktails were tested as described above. The results were evaluated visually by plotting a bar diagram that illustrated the measured R_e value for each screening cocktail. The stock solutions with the highest R_e values were chosen for further optimization.

In the second step, the two or three stocks with largest contribution to high R_e values were further investigated. Two different experimental designs for optimization were used. When two stocks were chosen for optimization, a two-dimensional mixture optimization design ("MO2D") was used. This design is illustrated in Figure 2, wherein the composition of the cocktails are denoted both in the table and the diagram. For those cocktails where the sum of volume percent was lower than 100%, water was added as a third solution. The evaluation of a two-stock optimization was visual. As exemplified in Figure 2, the calculated R_e values may be plotted in the corresponding positions in the design diagram.

When an optimization with three stocks was performed, a three-dimensional mixture optimization design ("MO3D") was used. Seven cocktails, as illustrated in Figure 3, were mixed, tested and assigned R_e values. The composition of the cocktails are denoted in the left hand boxes in the diagram. The evaluation of the three-stock optimization was also visual. As exemplified in Figure 3, the calculated R_e values may be plotted in the corresponding positions in the design diagram.

Results

The screening cocktails were designed to include diluted single stocks and all two part combinations of stocks. Of the 21 possible screening cocktails three had to be excluded: BIw and BOw formed precipitate, whereas scouting experiments showed that IO was too aggressive. That is, after an injection of an IO regeneration cocktail the maximal binding capacity was decreased and the baseline was increased significantly. Consequently, due to the necessity of gentle screening solutions, IO was excluded.

The RO protocol of the present invention was used to identify an optimized regeneration solution for four specific ligand-analyte systems: (1) p24/MAb 609 system; (2) p24/MAb 576 system; (3) GST/polyclonal anti-GST system and (4) SpA/MAbs.

(1) For the p24 MAb 609 system, several of the screening cocktails were identified as possible regeneration agents. The cocktail ICw seemed to offer complete regeneration in 30 seconds. AIw, AOw and DOw also gave high R_e in the screening session. Cocktails containing stock O caused an unstable baseline for approximately 30 seconds. The R_e measured for each screening cocktail is plotted in Figure 6. Control experiments showed that ICw only regenerated the surface close to completely. Thus, the cocktail AIC was successfully tested as regeneration agent for the p24-MAb609 system. In the control experiment, the MAb surface was regenerated 20 times with AIC. In Figure 7A, an overlay plot of cycle 1 and cycle 20 is shown. The plot clearly shows that the surface did withstand repeated injections of the cocktail AIC. (The p24-MAb609 system has been traditionally regenerated with 100mM HCl.)

(2) For the p24 MAb 576 system, ICw was also the best screening cocktail (ICw had a R_e>90). The R_e measured for each screening cocktail is plotted in Figure 8. Based on these results, an optimization with the stocks A, I and C was performed. In this experiment, a regeneration cocktail which offered complete

regeneration was identified. The cocktail AIC had $R_e \approx 100$ and was therefore identified as a suitable regeneration agent. The optimization results also indicated that all three components are needed to obtain complete regeneration, although the stock I contributes most to the regeneration properties of the cocktails. The results are shown in Figure 9. (The p24-MAb576 system is normally regenerated with 1M ethanolamine pH 10.4.)

(3) The regeneration effects for the screening cocktails for the GST- polyclonal antiGST system are plotted in Figure 10. The most successful cocktails, AIw and ICw had R_e values of approximately 60 and 45 respectively. Two stock solutions were chosen for optimization. A was chosen as the best stock solution and the mix I + (0.5M EDTA) (10+1) as second best. An optimization using the MO2D design was performed. In this design, not only was the volume percent of the acid stock solution varied, the pH of the stock was decreased with increasing volume percent as well. The results are plotted in Figure 11 (based on the MO2D format of Figure 2). Visual evaluation of the result plot show that one volume A with pH adjusted to 4 mixed with one volume I + (0.5M EDTA) (10+1) regenerated the surface completely. (The GST-antiGST is normally regenerated with a 10mM glycine solution pH 2.20.)

(4) In addition, to illustrate both the regeneration and characterization aspects of this invention, the RO protocol was applied to a fourth system - that is, SpA, as the ligand, with ten different antibody analytes: (a) g3_clone2, (b) g3_clone3, (c) g2b_clone2, (d) g2b_clone3, (e) g2a, (f) gla, (g) anti-theofyllin MAb 459, (h) anti-p24 MAb 609, (i) polyclonal anti-clenbuterol Abs r200 and (j) rl54. The resulting R_e values are plotted in Figure 12.

With regard to regeneration optimization, ICw and Alw had high regeneration effects (>50%) for all systems. Cocktails containing O had high regeneration effects for MAb 609 only. Also, r154 and r200 were regenerated close to completely by ICw and Alw, but were not especially affected by ADw, in contrast to g3_clone2 for which ICw, Alw and ADw all had high R_e.

As for characterization of the analytes, the regeneration pattern illustrated in Figure 12 may be used as a unique identification or "finger print" for each of the tested analytes. Such characterization may be used to predict the functionality of other analytes by comparison to a predetermined, test library of known molecules. For example, another MAb which exhibits similar characteristics to one (or more) of the above-tested MAbs would be expected to have similar activity to the known MAb. Such characterization has utility over a wide range of

applications, including (but not limited to) drug identification and/or optimization, quantitative assays, as well as epitope mapping.

Discussion of Results

The RO-protocol of the present invention is an extremely valuable tool for identifying and optimizing regeneration conditions that withhold full ligand activity, have high regeneration effect, offer quick recover to a stable baseline and work with short contact time. All experiments in the RO-protocol were designed to identify at least one cocktail that works. The RO-protocol of the present invention has several advantages over conventional regeneration optimization techniques. The protocol is based on multivariate experimental design which ensures maximal variation of the parameters under investigation as well as ease-of-use both regarding screening cocktail preparation and result evaluation. A validation of the regeneration results is also obtained by testing all cocktails in the design even if one is identified as possible regeneration agent early in the run. This is exemplified in Figure 9, where the complete result plot reveals that all three components are necessary, although the components influence to different extents.

The regeneration stock solutions were chosen with maximal diversity as primary goal. Most commonly used regeneration conditions can be obtained with cocktails of the stocks. The risk of damaging the ligand is low due to the gentle screening cocktails. The time spent for regeneration optimization is decreased, primarily because of the multivariate approach. In automated mode, the screening session may take 3-8 hours, depending on the biomolecular interaction system. Most important of all, the RO-protocol of the present invention works under true-laboratory conditions. The RO-protocol identified regeneration cocktails for many systems already in the first screening session. In two cases, an optimization was performed to identify suitable regeneration cocktail. The cocktail was optimized primarily towards 100% regeneration and secondly towards a quick return to a stable baseline. Furthermore, the RO-protocol provided a "map" over the system behavior for different regeneration conditions. This map can be very useful to start the manual investigation of regeneration conditions from if the RO-protocol cocktails fails to regenerate the surface. Furthermore, the screening cocktails can easily be made more concentrated by reducing the 33-67 volume percent water to 0-33% in all cocktails.

Previous work by van Oss (In *Immunochemistry*, van Oss and Regenmortel (Eds), Marcel Dekker, New York, 1994) indicates that combinations of agents of different kinds are preferable for breaking the binding between antibodies

and antigens, especially combinations of agents affecting the interactions between Lewis acids and Lewis bases (L) and electrostatic forces (E). For example, drastic pH decrease affecting mainly E combined with unpolar solvents affecting mainly L is a good regeneration agent in many cases. In the RO-protocol, such combinations are tested, but with two major differences compared to previous work: first, several different agents with similar main properties were used instead of combining one solvent with one acid/base; and, second, the combinations were chosen with a multivariate approach which minimizes the risk of missing relevant combinations of agents.

The above experiments illustrate that the cocktail approach used in the RO-protocol is successful. Several completely different cocktails had high R_e for some investigated systems. Also, the cocktails influenced the dissociation of the investigated systems to different extents. Collection of results for several systems show that multi-stock cocktails have significantly higher regeneration effects than single-stock cocktails. The screening cocktail AIw was expected to have better regeneration effects than Aww and Iww. The multivariate approach used in the RO-protocol revealed that another cocktail, ICw, had significantly higher R_e than those of the single diluted stocks Iww and Cww. The excellent regeneration properties of ICw was completely unexpected. This unexpected result exemplifies the strength of multivariate experimental design.

The sensorgrams must be evaluated correctly in order to give relevant information. Occasionally, matrix effects influences the sensorgram for several minutes. Most matrix effects are due to protein folding, but occasionally other mechanisms influences the matrix as well. The loss of detergents adsorbed to the surface and the use of the unpolar stock are two such examples. Carryover effects were prevented by extensive washes after each cocktail injection. Normally, signals due to different artifacts are transient and disappear within 60 seconds, with the exception of the detergent adsorption. It is important to learn to distinguish between true dissociation and signals due to artifacts. In the RO-protocol sensorgrams this is quite simple since systems with fast dissociation are unlikely to be subject to a regeneration investigation. Moreover, since an analyte injection was performed after each successful cocktail the surface status was continuously monitored.

When the analyte is a polyclonal antibody, the interpretation of the data may become more difficult. The R_e value will be too large when it is determined immediately after an analyte injection, and too low after a few analyte injections performed on incompletely regenerated surfaces. In spite of the difficulties in data

interpretation and the low R_e measured for the screening cocktails, only one optimization experiment was needed to identify a cocktail that regenerated the surface completely.

The ligand can also influence the signal in a confusing way. Some ligands need a few regeneration pulses to stabilize, which makes the determination of reference baseline difficult. This made the first few measured R_e values on some new surfaces less reliable. The suspiciously high R_e value 114 in the GST-antiGST optimization was probably due to such a baseline decrease. Negative R_e values also appear. These were probably due to matrix effects.

The simple evaluation plots, such as Figures 10 and 11, monitor trends in the behavior of R_e in a clear way. If the results from a session are unsatisfactory, the evaluation plot indicates which new stocks proportions that are favorable. Thus, the RO-protocol is a great help even if complete regeneration is not obtained. The visual evaluation also simplifies the identification of erroneous results ("outliers").

The RO-protocol identified two interesting combination effects which both seemed to be general. First, regeneration cocktail IOw was unexpectedly found to be too aggressive. Second, the stock I and C alone are significantly less good as regeneration agents than the cocktail IC. The only difference between the two solutions is the 6.7mM EDTA present in the latter solution. It should be noted that $[Mg^{2+}]$ = 610mM, which means that the EDTA-molecules are complexed with a Mg ion. Thus, the Mg^{2+}-EDTA complex, possibly in combination with either or both of the chaotropic agents GuHCI and urea and the SCN- ion, increases dissociation significantly. The optimization experiment for the GST system show that the effect of EDTA as additive was better at lower pH.

The results from the SpA experiments show that systems with one ligand and different analytes behave differently when the RO-protocol is applied to the systems. For example, only one system, SpA MAb 609, was affected by the cocktails Uww and UCw.

EXAMPLE 2

This example illustrates a representative method for the kinetic characterization of the interaction of Z-fragment of protein A (*i.e.*, one domain analogue of staphylococcal protein A) with mouse-IgG3 in a hypothetical volume in chemical space by use of a multi-buffer perturbation approach in accordance with the present invention. Specifically, the kinetic rate parameters for the interaction

between a one domain analogue of Staphylococcal protein A (hereinafter referred to as "Z") and mouse-IgG3 monoclonal antibody (Mab) were measured in HBS-buffer with different chemical additives. Five buffer ingredients (*i.e.*, pH, NaCl, DMSO, EDTA, KSCN) were simultaneously varied according to a statistical experimental plan in 16 experiments. The 16 buffers associated therewith spanned a volume in chemical space. A mathematical model using data from the various compositions of the 16 buffers was developed and used to predict kinetic parameters for five other buffer compositions that resided within the spanned volume of chemical space. The predicted kinetic parameters agreed with the subsequently measured values, thereby validating the mathematical model.

MATERIALS AND METHODS

Ligands, analytes and coupling to sensor chip

The ligand, mouse-IgG3, was a Biacore AB in-house reagent. The Z domain was produced in *E. coli* and IgG-affinity purified as described by Cedergren and coworkers (Cedergren et al., *Protein Engineering 6(4)*:441-448, 1993). Sensor Chip CM5, HBS running buffer (10mM Hepes, 150mM NaCl, 3mM EDTA and 0.005% Surfactant p20, pH 7.4) and Amine coupling kit (*N*-ethyl-*N'*-(dimethylaminopropyl)-carbodiamid (EDC), *N*-hydroxysuccinimide (NHS) and ethanoleamine-HCL pH 8.5) were also obtained from Biacore AB. The ligand was coupled to Sensor Chip CM5 in BIACORE 3000 according to Johnson and coworkers (Johnsson et al, *Journal of Molecular Recognition, 8(1-2)*:125-131, 1995). The CM5 sensor surface was activated with a 7 minutes EDC/NHS pulse. The ligand, dissolved in 10mM acetate buffer pH 4.8, was then injected for 3-5 minutes. Next a 7 minutes pulse of 1.0 M ethanolamine-HC1 pH 8.5 deactivated the surface. Immobilizations were performed at flow 10 µl/min and at 20°C. The immobilization levels for the ligands were typically 5000-7000 RU. A blank activated and deactivated carboxymethylated dextran surface was used as reference.

Buffers and experimental conditions

Buffers were prepared in accordance with the compositions of Table 6 (except that all of the buffers additional contained 10mM HEPES and 0.005% Surfactant P20. The measured results from buffers S1-16 were used to compute a mathematical model that allowed prediction of k_a and k_d values associated with other

buffer compositions. The S-buffers were chosen according to a fractional factorial design with resolution V. Such a design can resolve how both individual buffer ingredients and pairs of buffer ingredients affect the kinetics. For validation of the mathematical model, five buffers, denoted SQ1-5, were designed and formulated. The characterizations of the interaction of Z with IgG3 in the S-buffers and the SQ-buffers were performed on different, newly prepared sensor chips. Mathematical modeling and predictions were done in MODDE 4.0.

The kinetic characterizations were performed by injecting Z in three concentrations, ranging from 20 nM to 225 nM. The flow during the injections was 40 µl/min, the temperature was 20°C and the Z injections were one minute long. No regeneration was used, instead the interaction was allowed to dissociate in running buffer. To increase sample throughout a prototype inject command, "triinject," was used in this experiment. Triinject injects liquid A - liquid B - liquid A. If liquid A is a buffer and liquid B is a sample dissolved in the buffer, triinject makes the choice of interaction buffer independent from the choice of running buffer. BIAevaluation 3.0 was used to fit a 1-1 binding model to the data.

Table 6

	NaCl	KSCN	DMSO	pH	EDTA	Measured		Predicted	
	mM	mM	mM		mM	k_a (1/Ms)	k_d (1/s)	k_a (1/Ms)	k_d (1/s)
S1	150	2	20	7.2	23	110000	0.023		
S2	150	2	200	7.6	23	138000	0.0167		
S3	150	11	20	7.6	23	146000	0.0173		
S4	150	11	200	7.2	23	117000	0.0233		
S5	150	2	20	7.6	3	136000	0.018		
S6	150	2	200	7.2	3	105000	0.0252		
S7	150	11	20	7.2	3	122000	0.0259		
S8	150	11	200	7.6	3	142000	0.019		
S9	550	2	20	7.6	23	136000	0.021		
S10	550	2	200	7.2	23	105000	0.028		
S11	550	11	20	7.2	23	113000	0.0289		
S12	550	11	200	7.6	23	152000	0.021		
S13	550	2	20	7.2	3	118000	0.0307		
S14	550	2	200	7.6	3	131000	0.0218		
S15	550	11	20	7.6	3	142000	0.0208		
S16	550	11	200	7.2	3	115000	0.0312		
SQ1	157	11	20	7.6	23	13800	0.0187	$1.5*10^5$	0.016
SQ2	550	2	153	7.2	5	124000	0.0328	$1.1*10^5$	0.029
SQ3	164	2	110	7.2	23	125000	0.0251	$1.1*10^5$	0.023
SQ4	550	11	73	7.4	6.3	150000	0.0278	$1.3*10^5$	0.025
SQ5	350	6.5	110	7.4	13	149000	0.0255	$1.3*10^5$	0.022

Results

The kinetic parameters for the interaction of Z with IgG3 MAb were experimentally calculated for all buffers, as is further shown in Table 6. The kinetic parameters were then used to develop a mathematical model (S-model) that described the relation between S-buffers and the kinetic parameters. Because only a few buffer parameters influenced the kinetics as is shown in Figure 13, and because the chemical components did not significantly interact with one another, a very simple, linear model was sufficient for reproducing the observed data well, as set forth by Equations (2) and (3) below:

$$k_d = -0.019*[pH]+1.1*10E-5*[NaCl]-8.4*10E-5*[EDTA]+4.2*10E-5*[KSCN]+4.2*10E-7*[DMSO]+0.16 \quad (2)$$

$$k_a = 6.8*10E4*[pH]-1.3*[NaCl]+38*[EDTA]+9.7*10E2*[KSCN]-13*[DMSO]+3.8*10E5 \quad (3)$$

The S-model quality was estimated by cross-validation in MODDE 4.0. The calculated Q2 value was 0.95 for k_d and 0.85 for k_a, indicating that the S-model have good predictive power. The validation tool in SIMCA 7.0, which randomly reorders the results 20 times and tries to fit a model to the reordered data, gave no statistically significant models, which also indicates that the S-model is reliable. For further validation of the S-model, the kinetics for five additional buffers (SQ1-5) were experimentally determined and compared with the predicted values from the S-model. The values agreed thereby indicating that the model was valid.

In addition, the S-model was used for estimation of the error in each measurement. When comparing observed and predicted kinetic parameters for the S buffers, a maximal deviation of 10% was obtained, suggesting that all measurements have a typical error of less than 10%. This value was confirmed by comparing measured and predicted kinetic parameters for the SQ buffers, were k_d values had errors ranging from 8% to 14% and k_a values from -8% to 15%.

Discussion of Results

In general, an intermolecular interaction has two characteristic: (1) the event of recognition (association) and (2) the stability of the complex (dissociation). The presented data demonstrates that the interaction of Z with mouse-IgG3 MAb has a recognition that is more difficult to affect than the stability of the complex. In the volume of chemical space under examination, pH was the most important factor. Both association and dissociation were significantly affected by variations in pH, where the higher pH resulted in a stronger interaction. Dissociation was also clearly affected by NaCl, where a high concentration gave a less stable complex, whereas an increase of the EDTA concentration stabilized the complex. It was difficult to judge if the concentration KSCN significantly affected the association. The choice of DMSO concentration did not, however, influence the interaction at all. A diagram summarizing the effects of such varying concentrations is shown in Figure 15. (Note that it is well known that pH strongly affects the interaction of Z with IgG.)

With the detailed description of pH dependency provided by the S-model, the affinity of a biomolecular interaction can be adjusted to a specified level with high precision. The effects seen from an increased concentration of NaCl indicates that electrostatic forces are important only after the bond has been formed. The limited effect of EDTA may suggest a weak metal ion dependency but may also

be an effect of changed buffer capacity. The weak indication that the higher concentration KSCN might increase the association is interesting; if the KSCN effect is true, it suggests that limited disturbances of the water structure is preferable for recognition. The fact that DMSO does not influence the interaction probably indicates that the hydrophobic contributions to the binding are stable to minor perturbations.

The S-model, however, was less reliable for predicting association for two principle reasons: first, the determination of k_a is often less precise than k_d since k_d can be calculated without detailed knowledge about active concentration in the sample; and second, the largest and the smallest observed association parameter k_a differed by less than a factor 1.5. Thus, the association parameters have lower signal to noise ratio than the dissociation parameters, which makes it more difficult to develop mathematical models that can be used for predicting the association. Apart from pH, KSCN was the only candidate to be a statistically significant model term for the association. By performing more experiments, the increased resolution of the results would make it possible to determine the effects of KSCN more precisely. Since KSCN was the only parameter that was on the borderline, such experiments were not performed.

A convenient way to monitor the effects of important solution additives is to study sensorgrams. For example, overlay sensorgrams from the buffers giving the highest (*i.e.*, S8) and lowest (*i.e.*, S16) affinity are shown in Figure 16. The importance of knowing the dynamics of the kinetics for an interaction in the context of communicating kinetics in one buffer is obvious because of the limited differences between the buffers S8 and S16 causes the affinity of the interaction to decrease 50%.

A common way to estimate the reliability of the kinetic parameters of an interaction is to run replicates. This is straight-forward, but has several disadvantages. Most important is that the sensitivity to small changes in buffer conditions of the interaction in uncontrolled. Thus, when comparing the kinetics of two structural analogues in different preparations of the same buffer, for example, it is possible, by mistake, to misinterpret unexpected variations in buffer compositions for effects caused by structural differences. This can be avoided by performing a robustness test, where a few slightly modified buffers are used to estimate the interaction sensitivity to buffer compositions. If such a test shows that the buffer conditions do not influence the kinetics drastically, the kinetic parameters can be compared with confidence. However, if the robustness test shows that the interaction

is sensitive to changes in buffer composition a complete description of the kinetics in a volume of chemical space by using a mathematical model is preferable. Except for the kinetic parameters, it is possible to calculate the range of change associated with the parameters due to variations in buffer composition. It is also possible to predict kinetics in new buffers belonging to the investigated chemical space, and interpret the pattern of the results.

The validation with the SQ buffers demonstrates that it is possible to describe the kinetic dynamics in buffer space with precision similar to real measurements by mathematical modeling. In addition, the complete set of results provides more stable determinations of kinetic parameters. Thus, it is more appropriate to compare sets of kinetic parameters than results from a single buffer.

EXAMPLE 3

This example illustrates a representative method for the kinetic characterization of two Z-fragments of protein A with mouse -Ig63 in a hypothetical volume in chemical space by use of a multi-buffer perturbation approach in accordance with the present invention. Specifically, the interaction of Z and a mutated analogue of Z (Z6G) with monoclonal mouse-IgG3 were characterized in 16 different buffers. Measurements on Z are described herein; measurements on Z6G were performed in the same way - but only with the S-buffers (not with the SQ-buffers). Z6G has 6 extraglycine's between helix 2 and 3 compared to Z. The sequences for the binding-site region are set forth below (helices are underlined and IgG-binding amino acids are typed in bold):

Z ...VDNKFN KEQQNAFYEILH LPNLN EEQRNAFIQSLKD DPSQ SANLLAEAKKLNDA QAPK...
(SEQ ID NO:1)

Z6G ...VDNKFN KEQQNAFYEILH LPNLN EEQRNAFIQSLKD DPSQGGGGGGSANLLAEAKKLNDA QAPK...
(SEQ ID NO:2)

Discussion of Results

For Z6G, k_d for the interaction in the different buffers are summarized below in Table 7. The association was too fast to be quantified. A mathematical

model that could predict the dissociation parameter was developed (SZ6G-model) as expressed by Equation (4):

$$k_d = -0.15*[pH]+2.1*10E-5*[NaCl]-7.2*10E-4*[EDTA]+2.6*10E-4*[KSCN]+8.1*10E-6*[DMSO]+1.22 \quad (4)$$

The prediction power of the SZ6G-model was good (Q^2=0.83).

For Z, both association and dissociation parameters could be quantified. The dissociation parameter is also summarized in Table 7.

Table 7

Buffer	NaCl	KSCN	DMSO	pH	EDTA	k_d Z6G	k_d Z
s1	0	2	20	7.2	20	0.153	0.023
s2	0	2	200	7.6	20	0.102	0.0167
s3	0	11	20	7.6	20	0.106	0.0173
s4	0	11	200	7.2	20	0.155	0.0233
s5	0	2	20	7.6	0	0.109	0.018
s6	0	2	200	7.2	0	0.17	0.0252
s7	0	11	20	7.2	0	0.186	0.0259
s8	0	11	200	7.6	0	0.111	0.019
s9	400	2	20	7.6	20	0.017	0.021
s10	400	2	200	7.2	20	0.178	0.028
s11	400	11	20	7.2	20	0.154	0.0289
s12	400	11	200	7.6	20	0.125	0.021
s13	400	2	20	7.2	0	0.191	0.0307
s14	400	2	200	7.6	0	0.118	0.0218
s15	400	11	20	7.6	0	0.114	0.0208
s16	400	11	200	7.2	0	0.184	0.312

The model describing the relation between k_d and the buffer composition (S-model) is expressed as Equation (5):

$$k_d = -0.019*[pH]+1.1*10E-5*[NaCl]-8.4*10E-5*[EDTA]+4.2*10E-5*[KSCN]+4.2*10E-7*[DMSO]+0.16 \quad (5)$$

When comparing the interaction characteristics between Z and Z6G, there was one clear difference. When interacting with IgG3, Z6G has a k_d that is typically 10 times larger than Z. This difference can certainly be detected when studying the interaction in one buffer. The use of a set of buffers, however, reveals much more about the differences between Z and Z6G. When comparing the models in bar graph form as shown in Figures 17 and 18, it is clear that both interactions are sensitive to changes in pH. The relative sensitivity to pH is about the same. For Z,

the average k_d at pH 7.6 is 0.019 and at pH 7.2 is 0.027, corresponding to a 28% increase for k_d. For Z6G, kd increases 35% when changing pH from 7.6 to 7.2. The sensitivity to changes in concentration of NaCl is however different. For Z, increasing the NaCl concentration from 150mM causes k_d to decrease on average 21%. For Z6G, the same increase in NaCl concentration only decreases k_d on average 7%. Increasing the concentration EDTA decreases k_d about the same (7% for Z, 9% for Z6G). KSCN and DMSO do not significantly influence k_d for Z or Z6G.

The fact that the k_d of the Z-IgG3 interaction is significantly influenced by changes in the NaCl concentration is an indication that electrostatic forces are important for maintaining the complex between Z and IgG3. Z6G has on average a higher k_d (*i.e.*, a weaker binding) and is significantly less sensitive to changes in NaCl concentration compared to Z. This indicates that the reason for the weaker interaction Z6G is displacement of charged groups in the binding-site due to the mutation in Z6G. Detection of the different sensitivity pattern due to different chemical additives is not possible unless one uses several buffers. The interpretation of differences in sensitivity pattern, as in the example above, are important when studying the reasons for differences in binding characteristics are performed.

EXAMPLE 4

This example illustrates a representative method for the characterization of reagents for quantitative assays with respect to stability in the sample material by use of varied buffer solutions. Specifically, the properties of a reagent (antibody) used in a quantitative biosensor assay should include high affinity for the analyte and high stability in the sample matrix, *i.e.*, minimal influence on the reagent by variations between samples of the same matrix. Affinity is easily tested by running calibration curves and calculating IC_{50} for each reagent. However, there is no simple way to determine the stability of the reagent in a certain matrix. The evaluation of stability in matrix usually starts at a latter stage in assay development, often after one reagent has already been picked and used for some time. The stability (or instability) might not be fully determined until a large number of real samples have been tested (and the diversity of matrix composition has been fully covered with the samples run). If the reagent at this stage fails to fulfill the requirements a lot of work has been done for no good. It would be highly convenient if it were possible to

choose in a simple way between several reagents with respect to stability in matrix at an initial stage of assay development, without the need for real samples.

The disclosure of this Example demonstrates the ability to predict stability in sample matrix of a reagent by using designed artificial buffer samples instead of real samples. A number of reagents were characterized in both artificial buffer samples and in real samples. The antibiotin-biotin system (an inhibition assay system with biotin on the surface) was used as model system. The system gave access to several reagents (antibiotins) and a large number of real samples (18 infant formulas and cereal products). Four reagents of different makes were tested.

A set of eight buffers were prepared according to a multivariate design (seven factors on two levels) and used as controlled artificial samples. The four reagents were diluted in the eight buffers and spiked with biotin at four levels. The measured response of the spikes from each buffer was plotted in the same graph and the standard deviation of the relative response was calculated for each of the spiked concentrations. Correspondingly, the reagents were run in infant formula samples. To obtain a heterogenous set of real samples, the infant formulas were picked so as to fit the corners of a cubic experimental design. The factors in the design (the three axes in the cube) were chosen to correspond to three distinct variables in the formula composition: (1) presence of soy protein or not, (2) fat content; and (3) pH in the ready-mixed beverage. The infant formulas were spiked at three levels with biotin and run with the four reagents. The measured response of the spikes were plotted in the same graph and the standard deviation of the relative response was calculated for each of the spiked concentrations. The results from the artificial samples and the real samples were compared and the four reagents were evaluated accordingly.

MATERIALS AND METHODS

Apparatus and Materials

BIAcore™ 2000, id CA 2001 and 1001
Sensor Chip CM5
NHS, Biacore
EDC, Biacore
Etanolamine-HCl, Biacore
HBS-EP, Biacore
Biotinhydrazide, Sigma
Biotin, Sigma
2-[4-(2-hydroxyethyl)-1-piperazinyl]etanesulfonic acid (HEPES), Merck
Sodiumchloride, Merck
EDTA disodiumsalt, Merck
Surfactant P20, BIACORE
Ethylenglycol, Riedel-de Haën
Bovint Serum Albumine, Pharmacia Diagnostics
Millipore water

Instrument settings (BIAcore™ 2000)

temperature	-	20°C
flow	-	20 µl/min
flowbuffer	-	HBS-EP

Antibiotin, working concentration and regeneration conditions

Four different antibiotin were used in the study. The working concentrations used correspond to approximately 1200 RU maximum binding signal. The regeneration conditions were optimized for each of the four reagents. (See Table 8 below, wherein ROKS solution I is a solution of KSCN (0.46M), MgCl2 (1.83M), urea (0.92M), guanidinHCl (1.83M) and ROKS solution U is a mix of equal volumes of DMSO, formamide, ethanol, acetonitrile and 1-buthanol).

Table 8

Make	Specification	Working concentration	Regeneration condition
Novocastra	Monoclonal Mouse Anti-biotin	3.3 µg/ml	30 s 20 mM NaOH, 0.005% Surfactant P20
Goldmark	Monoclonal Mouse Anti-biotin	1.6 µg/ml	30 s ROKS solution IU* 3*15 s 200 mM NaOH, 0.005% Surfactant P20
Sigma	Monoclonal Mouse Anti-biotin	5.0 µg/ml	30 s 20 mM NaOH, 0.005% Surfactant P20
Pierce	Immunopure Goat Anti-biotin	2.4 µg/ml	30 s ROKS solution IU* 3*15 s 200 mM NaOH, 0.005% Surfactant P20

Assay principle

The antibiotin-biotin assay is a so-called inhibition assay. Biotin is immobilized onto the sensor surface and antibiotin is added in surplus to the sample of interest before run in the BIAcore instrument. In this study, biotinhydrazide chip surfaces were used. Biotinhydrazide was immobilized on CM5 surfaces with a conventional amine-coupling technique. The immobilization was performed outside the BIAcore instrument to avoid contamination of tubings and IFC. For analysis, the reagent was diluted in buffer solution to four times the working concentration and mixed 1+3 with biotin standard solution or sample filtrate. Sample injection volume: 30 µl.

Artificial sample environment

In this experiment, a fractional factorial design, resolution III, was used. Specifically, seven factors were varied on two levels in eight experiments (*i.e.*, eight buffer solutions). The factors were chosen to reflect possible variations in the sample matrix. The factors and the levels are presented below in Table 9. The compositions of the eight buffer solutions are listed below in Table 10. HBS-EP was used as reference buffer and center point experiment in the design.

Table 9

	factor	low		high	unit
1	NaCl	0.10	-	0.50	M
2	BSA	0	-	1	%(w/v)
3	HEPES	10	-	50	mM
4	pH	5.9	-	7.4	
5	Ethyleneglycol	0	-	1	%(v/v)
6	Surfactant P20	0.001	-	0.009	%(v/v)
7	EDTA	0	-	6.8	mM

Table 10

buffer no.	NaCl (M)	BSA (%(w/v))	HEPES (mM)	pH	Ethylene-glycol (%(v/v))	Surfactant P20 (%(v/v))	EDTA (mM)
1	0.1	0	10	7.4	1	0.009	0
2	0.5	0	10	5.9	0	0.009	6.8
3	0.1	1	10	5.9	1	0.001	6.8
4	0.5	1	10	7.4	0	0.001	0
5	0.1	0	50	7.4	0	0.001	6.8
6	0.5	0	50	5.9	1	0.001	0
7	0.1	1	50	5.9	0	0.009	0
8	0.5	1	50	7.4	1	0.009	6.8

All buffer solutions were made four times stronger than the final (working) concentration (as shown in Table 10). To facilitate the preparation, BSA was added as a 0.1g/ml water solution. If necessary, the solutions were heated carefully to solve all material. The solutions were vacuum filtered through 0.45 µm filters.

The reagents were diluted in the eight artificial buffers and a 4*HSB-EP solution (four times more concentrated) to four times the working concentration. The antibody-buffer solutions were spiked with biotin by mixing 50 µl antibody-buffer solution with 150 µl biotin calibration solutions 1.37, 12.35, 111.11 and 1000 ng/ml, respectively. Sample triplicate of the HBS-EP buffer (*i.e.*, center point experiment) were prepared. The samples were run in random order. Injection triplicate of HBS-EP were evenly spread over the run time to obtain control over the drift in response with time.

Infant formula samples

A full factorial screening design with three factors at two levels was picked for this experiment. This design is easily visualized as a cube having one factor represented by each of the three axes. Eight experiments, corresponding to different infant formula samples, were needed to cover the corners of the cube.

Three distinct variables in the infant formula composition were chosen as factors: (1) presence of soy protein or not, (2) fat content; and (3) pH in the ready-mixed beverage. Soy protein in food samples has shown to in some instances interact with the reagent used in the assay. Many infant formulas and other cereal products contain soy protein. A reagent used in an assay for these kinds of products should preferably not be affected by such variations. There are large differences in pH between different ready-mixed formulas, ranging from approximately 4.8 to 7.3 in the infant formula samples measured. There are also relatively large variations in the fat content between different formulas. Formulas intended for pre-natal have the highest fat content, 25-30g/100g, whereas other formulas contain less than 10g/100g.

In this study 18 different infant formula samples and cereal products were available. It was, however, not possible to find one infant formula to fit each of the eight corners of the experimental cube. VeteDiet, a diet cereal product, was therefore used as one of the samples. Also, in three of the samples, pH was adjusted to fit the last three corners. The infant formula samples used in the experiment and the levels of the three factors are listed below in Table 11.

Table 11

No.	Design Matrix	Sample	Soy Protein	Fat (g/100g)	pH
1	- - -	LactoPlusMalt pH adjusted to 5	-	7.0	5.0
2	+ - -	VeteDiet pH adjusted to 5	Yes	8.5	5.0
3	- + -	Pelagon	-	22	4.9
4	+ + -	Alsoy pH adjusted to 5	Yes	25	4.9
5	- - +	Sinlac	-	9.0	7.1
6	+ - +	VeteDiet	Yes	8.5	6.9
7	- + +	Prebeba	-	26	7.3
8	+ + +	Alsoy	Yes	25	7.1

Infant formulas are fortified with low levels of biotin. The levels are equivalent to 1-2 ng/ml on the biotin calibration curve. Spike levels lower than 10 ng/ml are therefore not advisable. The spike levels were chosen so that one of them would lie close to IC_{50} (*i.e.*, in the steepest part of the curve) in the calibration

curve for each of the reagents. Three spike levels were chosen; 10, 40 and 100 ng/ml. Biotin spike solutions were prepared ten times more concentrated than the final concentration; 100, 400 and 1,000 ng/ml.

In preparing the infant formula samples, the sample preparation protocol for the BiacoreQuant Biotin Kit (Biacore AB, Uppsala, Sweden) was followed; however, minor adjustments were made as appropriate.

1.00-1.09g sample aliquots were weighed into 50 ml-vials. Three aliquots per sample. Each of the three sample aliquots were spiked with biotin by adding 5.0 ml 100, 400 and 1000 ng/ml biotin standard solution respectively (giving a final biotin concentration of 10, 40 and 100 ng/ml). 30 ml water was added to the vials. The samples were thoroughly mixed and then let to swell for 1h. The samples were quickly mixed before pH was measured. The pH was adjusted in sample nos. 1, 2 and 4. To reach a stable pH level, the samples were allowed to equilibriate 3-4 times between the adjustments. The samples were quantitatively transferred to 50 ml volumetric flasks and diluted. Approximately 3 ml-aliquots were transferred to centrifuge vials and spun for 20 min at 3000g. The supernatant was filtered through 0.22 filter and the filtrate was collected for analysis.

In preparing the samples for analysis, the four reagents were diluted in 4*HBS-EP to four times the final working concentration. 50 µl diluted reagent was mixed with 150 µl infant formula filtrate. Sample triplicate of sample no. 1 (all three concentrations) were prepared. The samples were run in random order along with biotin spikes 1.37, 12.35, 111.11 and 1000 ng/ml for each reagent. Injection triplicate of sample no. 1 were evenly spread over the run time to obtain control over the drift in response with time. Sample injection volume was 30 µl.

Discussion of Results

The relative response values obtained in the artificial buffer samples were plotted together with the calibration curve against biotin spike concentration. One plot for each reagent. The same was done for the spiked infant formulas. Figures 19A-D show spikes in the artificial buffer samples and Figures 20A-D show spikes in infant formula samples.

The plots well describe the spread in response for the different reagents. In the artificial samples, the antibiotins from Novocastra and Sigma give a small spread in response, whereas Goldmark and Pierce give a large spread in response. The same pattern for the reagents is seen in the real samples. In other words, there is large agreement between the data obtained in the artificial buffer

samples and the data obtained in the real infant formula samples. From these results, the conclusion drawn is that it is fully satisfactory to perform measurements in designed artificial buffer samples to get adequate information for determining the best reagent for a quantitative assay regarding stability in sample matrix.

To verify the conclusions from the visual evaluation, the results were also evaluated statistically. Standard deviation (std) of the spread in response was calculated for each spiked concentration. The sum of the standard deviations at each biotin concentration was calculated for each reagent in the artificial samples and real samples, respectively, and plotted against each other in a graph. This was done for raw data as shown in Figure 21, and also for normalized data as shown in Figure 22. The calibration curves in the different runs start on slightly different R_{max} (*i.e.*, the average response for the lowest biotin spike concentration, 1.37 ng/ml) and the normalization adjusts the plots to the same scale. The R_{max} was set to 100 units and 0 RU to zero units. If the spread in response is dependent on the R_{max} this would be a more correct evaluation of the data. However, the spread between samples is due to variations in sample composition, and it might be incorrect to subject normalization to such data. In that case, the evaluation from raw data would be the more correct. In any case, the two plots show the same trend; a small spread in response in the real samples also give a small spread in the artificial samples and vice versa - confirming the conclusion from the visual evaluation.

For the test system used in this assay (the antibiotin-biotin system), there was only one reagent that may be fairly regarded as the best choice. The Novocastra antibiotin is the only reagent that fills the sensitivity (affinity) requirement, which for a quantitative assay is a very important factor. In addition, the Novocastra antibiotin is satisfactory in terms of matrix stability. (Note that if the affinities of the four reagents had been closer, the antibiotin from Sigma, the second of the two "high stability" reagents, may also have option for this system).

Accordingly, this example clearly shows that by performing measurements in designed artificial buffer samples, it is possible to predict which reagent is best suited to be used in a quantitative assay with respect to stability in sample matrix without the need for real matrix samples.

EXAMPLE 5

This example illustrates a representative method for obtaining more information about the nature of the biomolecular interaction of Example 2 by use of a

multi-buffer perturbation approach in accordance with the present invention. More specifically, when performing structure-function relationship investigations, a number of similar molecules are synthesized and their ability to bind to one target is characterized. One way of obtaining a characterization of the binding is to use an affinity-based biosensor, such as BIACORE® 3000. By using such instrumentation, the interaction between each molecule and the target can in many cases be characterized by measuring the association rate and dissociation rate (*i.e.*, k_a and k_d). However, the measured values of k_a and k_d seldom reveal the reasons for why the molecules bind in the way they do.

In this example, the multi-buffer method discussed in Example 2 was applied to twelve similar peptides binding to one FAb. The multi-buffer method revealed that the peptides could be clustered in groups according to how they were perturbed. This new characterization did not correlate with the ordinary k_a and k_d ranking. Thus, by including perturbation pattern in a characterization of interactions, the resolution increases. It was also possible to develop a quantitative structure - buffer perturbation relationship (QSPR). Such a QSPR is a complement to ordinary quantitative structure - activity relationships (QSAR). By combining QSPR and QSAR more information about the nature of the molecular interaction is obtained.

MATERIALS AND METHODS

Buffer-Kinetics relationships

Recombinant antibody fragment (FAb) 57P was produced at IBMC (Strasbourg, France) (Chatellier, J., Rauffer-Bruyère, N., van Regenmortel, M.H.V., (1996) *J. Mol. Recognit. 9*:39-51). This FAb interacts with the residues 134-146 of the tobacco mosaic virus protein (Al moudallal, Z., Briand, J.P., van Regenmortel, M.H.V. (1985), *EMBO J. 4*:1231-1235). Twelve synthetic peptides, 16 or 19 residues long, were designed using the wild type protein as template (Table 12). An N-terminal cystein was added to allow coupling to CM5 sensor surfaces. The peptides were immobilized to sensor chip CM 5 following the instructions in the BIAapplications handbook (Biacore, Uppsala, Sweden). In all experiments an unspecific peptide was immobilized in flowcell one to make possible a subtraction of a reference sensorgram. The Fab was injected for 120 s at flow 30µl/min using the TRIINJECT command in three concentrations ranging from 6nM to 160nM diluted in the designed set of P-buffers (Table 13) and HBS (10 mM HEPES, 150 mM NaCl,

0.005% Surfactant P20, 3.4 mM EDTA, pH7.4). Kinetic parameters were calculated in BIAevaluation 3.0. For each peptide, a buffer-kinetics relationship (BKR) was calculated in MODDE 4.0 (Umetrics AB, Umeå, Sweden) using log10(k_a) and k_d in all P-buffers as response variables (Table 15). Three peptides were characterized two times for estimation of reproducibility. To make the different BKRs comparable, the average effect on a kinetic parameter of increasing a buffer parameter from its low value to its high value expressed in percent of the center point P-buffer (average of buffers P17, P18 and P19) was used. In practice, a relative perturbation of +10% for NaCl perturbing k_d means that in general, k_d increases 10% relative to k_d for the center point buffer when the NaCl concentration is increased from the center point value to its high value (for P-buffers 350mM to 550mM) and k_d is decreased 10% relative to the center point buffer when the NaCl concentration is decreased from the center point value to its low value (for P-buffers 350mM to 150mM). The relative perturbation not only make it easier to compare the different BKRs, it is also a value that may be more easily understood.

Quantitative sequence-perturbation relationship

The twelve peptides were described using a number of published descriptors (Table 16, wherein four different descriptors were used to characterize each position; the first three (a,b,c) correspond to the z-scale (a=zz1, b=zz2, c=zz3), where a is hydrophobicity, b is MW/surface area and c is electronic properties; the fourth descriptor, h, is a thermodynamic scale for the helix forming tendencies of amino acids (ΔG° when unfolding designed peptides with urea)). The parameters sum_a, etc. are the sum of the descriptor values for all three positions.) (O'Neil, K. T. DeGrado, W.F., "*A Thermodynamic Scale For The Helix Forming Tendencies of the Commonly Occurring Amino Acids*," (1990), *Science 250*:646-651; Sandberg, M., Eriksson, L., Jonsson, J., Sjöström, M., Wold, S., "*New Chemical Descriptors Relevant for the Design of Biologically Active Peptides. A Multivariate Characterization of 87 Amino Acids*,"(1998) *J. Med Chem.* 41:2481-2491). A mathematical model, quantitatively describing the relationship between peptide descriptors and relative perturbation was developed in SIMCA 7.0 (Umetrics AB, Umeå, Sweden) using PLS.

Discussion of Results

By plotting the kinetic parameters k_a versus k_d, both measured in HBS buffers (Table 14), an overview of how the different peptides bind to the FAb is

obtained (Figure 23). The buffer-kinetics relationships (Table 14) showed that the interactions were primarily perturbed by NaCl, urea and DMSO during association and by NaCl, urea, DMSO and EDTA during dissociation. By plotting the relative perturbation of k_a by NaCl and DMSO (the two parameters influencing association most) versus each other, a pattern showing the association characteristics is obtained (Figure 23). The relative perturbation of k_d by NaCl and DMSO is presented in the same way (Figure 24). By comparing Figures 23, 24 and 25, it is clear that the buffer perturbations are not correlated with k_a and k_d. The buffer perturbation data can be used in at least three different ways: (1) for increasing the resolution in a quality control assay; (2) to increase the information obtained in structure-activity analyses; and (3) when suggesting hypotheses on reasons for intermolecular interactions.

First, in a quality control situation, the buffer perturbation method can be valuable to differ similar molecules from each other. Suppose that DRK is produced. Controlling quality of synthesis by measuring k_a and k_d in one standard buffer, such as HBS, would introduce a risk of classifying VQE and QDF as correct product. However, if a plurality of buffers are used and a BKR is obtained, DRK, VQE and QDF can be clearly separated (Figures 24 and 25).

Second, Figure 22 shows that the different peptides bind differently to the FAb. For example, SAS binds fast and dissociates slow, GSQ binds fast and dissociates fast, FGR binds slow and dissociates fast and DRK binds slow and dissociates slow. Sensorgrams for these peptides are shown in Figure 26. The twelve peptides constitute a good dataset for structure function analysis because the peptides are similar, they all bind to the same target and they also have differences in binding strength. However, a structure function analysis will gain from additional information from the perturbation analysis. In this experiment, three buffer parameters that significantly perturbed the interactions were identified: DMSO perturbing k_a and k_d, as well as NaCl perturbing k_d. A mathematical model was developed to describe how the properties of each mutated residue of a peptide (Table 16) was related to perturbations due to those additives in the buffers (Table 17). The model quality was good for all three responses ($0.86>R^2>0.80$, $0.75>Q^2>0.58$) (Figures 27a-c). From coefficient plots (Figure 28a-c) it is possible to determine the importance of the different parameters. For example, the mathematical model relating NaCl perturbations on k_d to sequence descriptors has low coefficients for amino acid descriptors for positions 142 and 146 - thus these amino acids are not primarily involved in an electrostatic interaction. Position 145 descriptor a and the square of the sum of descriptor c have large coefficients. 145a has a positive

coefficient which means that the relative perturbation increases upon an increase of parameter 145a. That is, when the amino acid 145 becomes more hydrophobic, the interaction dissociates faster. The strong dependency of overall electrostatic properties (squared sum_c) indicate that a very high or very low sum of descriptor c for all positions increases the sensitivity to changes in NaCl concentration. Thus, if the goal is to obtain an interaction that is not sensitive to variations in NaCl concentration, a hydrophilic amino acid should but put in position 145 and the sum of descriptor c for all three positions should be between -2 and 3 (Figure 29).

Third, when comparing the interaction of the peptides DRK, DSA and DYD with FAb 57P, different reasons exist for the different binding characteristics. DYD and DSA have similar binding characteristics, while as DRK binds less fast and less strong. By comparing the amino acid descriptors for position 145 and 146, descriptor c for both positions in DRK are deviating most from DYD and DSA (Table 18). Thus, the reason for DRK binding more poorly than DYD and DSA might be improper electronic properties in position 145 and 146.

By analyzing the perturbation pattern for DRK, DYD and DSA, this hypothesis was confirmed. The descriptor c correspond to electronic properties, that include both charge and polarity. If the electronic properties are changed at a position where it is important to have proper electronic properties, the sensitivity to additives affecting polarity and charge should change. DRK is more sensitive to variations in NaCl concentration and less sensitive to variations in DMSO concentrations than DYD and DSA (Figure 30). Thus, the hypothesis is correct. Furthermore, DRK has a clear EDTA dependence that DYD and DSA do not have. This might indicate that DRK binds weak enough to be disturbed by metal ions, a perturbation that a normal binding withstand.

Table 12

Abbreviation	Sequence
wild type	NH- - CGSYNRSSFESSSGLV - CONH SEQ ID NO:3
VQE	NH- - CGSYNRVSFQESSGLV - CONH- SEQ ID NO:4
DYD	NH - CGSYNRDSFYDSSGLV - CONH- SEQ ID NO:5
GRA	NH - CGSYNRGSFRASSGLV - CONH- SEQ ID NO:6
GSQ	NH - CGSYNRGSFSQSSGLV - CONH SEQ ID NO:7
FGR	NH - CGSYNRFSFGRSSGLV - CONH- SEQ ID NO:8
QDF	NH - CGSYNRQSFDFSSGLV - CONH- SEQ ID NO:9
RDG	NH- - CGSYNRRSFDGSSGLV - CONH SEQ ID NO:10
DRK	NH- - CGSYNRDSFRKSSGLV - CONH SEQ ID NO:11
DSA	NH - CGSYNRDSFSASSGLV - CONH- SEQ ID NO:12
NES	NH- - CRGTGSYNRNSFESSSGLV - CONH- SEQ ID NO:13
SEA	NH - CRGTGSYNRSSFEASSGLV - CONH SEQ ID NO:14
SAS	NH - CRGTGSYNRSSFASSGLV - CONH- SEQ ID NO:15

Table 13

Name	NaCl	KSCN	DMSO	pH	EDTA	urea
P1	150	4	30	7	3	40
P2	150	4	300	7	23	400
P3	150	22	30	7	23	400
P4	150	22	300	7	3	40
P5	550	4	30	7	23	40
P6	550	4	300	7	3	400
P7	550	22	30	7	3	400
P8	550	22	300	7	23	40
P9	150	4	30	7.8	3	400
P10	150	4	300	7.8	23	40
P11	150	22	30	7.8	23	40
P12	150	22	300	7.8	3	400
P13	550	4	30	7.8	23	400
P14	550	4	300	7.8	3	40
P15	550	22	30	7.8	3	40
P16	550	22	300	7.8	23	400
P17	350	13	165	7.4	13	220
P18	350	13	165	7.4	13	220
P19	350	13	165	7.4	13	220

Table 14

Peptide	Kinetic parameters in HBS buffer		Kinetic parameters in buffers P17 P18 P19 (average)		Association. Effect on log10(ka) of increasing a buffer parameter from low to high value expressed as percent of average log10(ka) for buffers P17 P18 P19.						Dissociation. Effect on kd of increasing a buffer parameter from low to high value expressed as percent of average kd for buffers P17 P18 P19.					
	Ka	kd	ka	kd	NaCl	DMSO	urea	KSCN	EDTA	pH	NaCl	DMSO	urea	KSCN	EDTA	pH
error	1/Ms	1/s	1/Ms	1/s	%	%	%	%	%	%	%	%	%	%	%	%
estimation	22%	8%	22%	8%	±1.15	±0.30	±0.50	±0.58	±0.57	±0.07	±2.72	±3.60	±4.13	±1.16	±5.56	±0.75
VQE	3.71E+05	4.82E-03	1.18E+05	5.51E-03	-5.6	-3.2	-2.5	1.0	0.5	-0.2	-2.1	39.1	18.3	2.4	-5.3	0.1
DYD	5.29E+05	1.32E-02	1.80E+05	1.88E-02	-6.7	-2.4	-3.3	-0.9	-0.4	-0.9	-12.3	38.1	17.7	1.3	-4.1	-5.6
DYD	6.74E+05	1.50E-02	2.17E+05	1.80E-02	-5.8	-2.5	-2.9	-1.5	-1.1	-0.8	-16.2	32.7	12.0	0.4	-1.9	-6.5
GRA	8.16E+05	5.05E-03	3.33E+05	7.86E-03	-3.6	-2.2	-3.0	-0.8	-0.6	-0.4	-11.9	41.3	21.9	4.7	-2.4	-3.3
GSQ	7.95E+05	3.25E-02	3.24E+05	4.91E-02	-3.1	-3.0	-2.9	-1.1	-0.4	-0.4	3.1	30.6	24.4	8.2	-3.3	-4.9
FGR	2.98E+05	4.76E-02	1.35E+05	9.07E-02	-4.8	-3.2	-4.2	-0.3	-1.2	-0.3	-0.5	22.1	23.2	4.0	2.5	2.6
QDF	3.96E+05	4.91E-03	1.70E+05	6.54E-03	-4.7	-2.4	-3.1	-0.8	0.2	-0.6	10.8	26.1	10.6	10.8	-0.1	2.7
RDG	7.35E+05	1.21E-02	2.40E+05	1.60E-02	-4.3	-3.8	-3.0	-1.0	-0.4	-0.4	10.8	18.1	16.4	8.5	-3.3	-2.9
RDG	6.81E+05	1.22E-02	3.12E+05	1.71E-02	-3.6	-3.5	-3.0	-0.5	-0.5	-0.3	10.1	15.2	15.1	7.7	-5.1	-2.0
DRK	3.68E+05	3.84E-03	1.67E+05	4.51E-03	-3.4	-3.4	-3.4	-0.4	0.3	0.2	-19.8	18.2	18.6	4.6	-15.0	0.1
DSA	5.55E+05	1.34E-02	2.26E+05	1.46E-02	-3.6	-2.3	-2.8	-0.4	-0.2	-0.3	-11.0	25.7	18.2	5.6	-1.8	1.3
NES	5.69E+05	1.78E-03	2.05E+05	2.43E-03	-5.2	-1.9	-2.7	-1.3	-1.1	-1.0	8.3	30.7	21.7	0.5	-10.2	-1.5
NES	5.03E+05	1.70E-03	1.75E+05	2.60E-03	-6.9	-2.3	-3.5	-0.6	-0.5	-1.0	5.8	31.5	17.6	2.1	-1.0	-1.2
SEA	7.77E+05	7.96E-04	2.74E+05	1.07E-03	-4.9	-2.2	-2.8	-1.2	-0.4	-0.6	8.8	25.6	22.7	2.8	-5.3	0.8
SAS	1.03E+06	5.31E-03	3.93E+05	7.89E-03	-4.9	-2.3	-2.5	-1.1	-0.9	-0.3	7.1	35.5	20.8	3.8	-1.0	0.1

Table 15

	DRK		DSA		DYD				SAS		FGR		GRA		GSQ		QDF		RDG				NES				SEA		VQE	
	ka $*10^5$	kd $*10^{-3}$	ka $*10^5$	kd $*10^{-2}$	ka1 $*10^5$	ka2 $*10^5$	kd1 $*10^{-2}$	kd2 $*10^{-2}$	ka $*10^5$	kd $*10^{-3}$	ka $*10^5$	kd $*10^{-2}$	ka $*10^5$	kd $*10^{-3}$	ka $*10^5$	kd $*10^{-2}$	ka $*10^5$	kd $*10^{-3}$	ka1 $*10^5$	ka2 $*10^5$	kd1 $*10^{-2}$	kd2 $*10^{-2}$	ka1 $*10^5$	ka2 $*10^5$	kd1 $*10^{-3}$	kd2 $*10^{-3}$	ka $*10^5$	kd $*10^{-3}$	ka $*10^5$	kd $*10^{-3}$
P1	3.12	4.32			4.91	6.77	1.64	1.66	9.72	5.74	3.37	6.57	7.25	6.45	7.36	3.55	3.64	5.19	6.35	5.34	1.36	1.34	7.82		1.80		7.41	0.807	3.35	4.46
P2	1.46	5.25			2.47	2.75	2.45	2.38	4.99	9.12	1.31	10.4	3.40	10.3	4.39	6.05	1.91	6.77	2.58	2.58	1.71	1.67	3.05	2.26	2.72	2.90	4.00	1.15	1.59	6.60
P3	1.76	4.91			2.75	2.66	1.99	1.92	5.81	7.96	2.05	9.65	3.48	8.19	3.94	5.22	2.33	6.33	3.21	3.36	1.66	1.55	2.99	2.46	2.26	2.43	4.11	1.05	1.95	5.35
P4	2.08	5.63	3.59	1.62	3.35	3.91	2.19	2.20	6.60	8.15	2.34	8.54	4.99	9.00	4.56			7.07	3.21	3.57			3.70	3.47	2.59	2.64	4.91	1.06	2.09	5.41
P5	1.80	3.29	2.69	1.07		2.52		1.39	4.73	6.44	1.65	6.86	4.06	5.45	4.98	3.86	2.00	5.88	2.72	3.35	1.44	1.37	2.26	2.00		2.08	3.32	0.905	1.58	4.71
P6	0.87	5.10	1.48	1.61		1.31		2.07	3.30	1.01	0.90	10.2	2.01	9.85	2.62	6.44	1.01	7.86	1.36	1.67	1.95	1.88	1.85	0.96	3.31	3.34	2.18	1.40		
P7	1.14	4.99	1.89	1.37	1.34		1.80		3.26	8.12	0.95	8.39			3.53	5.51	1.37	7.35	1.95	2.22	1.84	1.81	1.76	1.22	2.86	2.66	2.44	1.17	0.92	5.55
P8	1.30	3.55	1.92	1.40	1.64	1.51	1.93	1.89	3.37	8.70			2.68	8.19	3.31	5.14	1.50	7.57	1.94	2.23	1.77	1.72	2.03	1.39			2.45	1.09	1.05	6.14
P9	1.85	5.26	2.70	1.55	2.71	2.98	1.78	1.69	7.08	7.69	1.83	9.23	3.81	7.69	4.58	4.36	2.04	6.09	3.13	3.23		1.59		2.48		2.37	3.97	1.11	1.43	5.39
P10	2.08	4.76	3.31	1.49	2.96	3.19	1.92	1.92	7.08	7.69	1.72	8.54	4.32	8.24	4.98	4.21		6.72	3.15	3.34	1.39	1.33		2.69		2.51	5.12	1.01	1.77	5.86
P11			3.45	1.30		3.30		1.46			2.76	7.44	4.51	6.20	6.29	3.68		6.38	3.77	4.33	1.32	1.34			1.76	2.09				
P12	1.27	6.41	2.18	1.90	1.99	2.16	2.37	2.21			1.43	11.0	3.20	10.6	3.53	6.18	1.54		2.13	2.59				1.71		2.97	2.85	1.33	1.54	8.30
P13	1.38	3.97	1.88	1.35	1.14	1.46	1.49	1.39	2.95	7.69		9.34	2.48	6.29	3.10	4.47	1.25	6.67	1.98	2.19	1.60	1.60	1.52	1.08	2.39	2.44	2.27	1.09	0.87	4.70
P14			2.00	1.37	1.37	1.66	1.81	1.67	3.62	8.74	1.60	8.58	2.89	7.18	3.61	4.61	1.57	7.42	2.14	2.44	1.60	1.57	1.96	1.23	2.74	2.60	2.69	1.11	0.90	6.01
P15	1.82	3.77	2.47	1.20	1.92		1.24		4.58	6.39	1.65	7.75			4.23	3.86	1.39	6.42					2.23	1.59	1.86	2.09	3.03	0.939	1.63	4.29
P16	1.04	4.54	1.37	1.73	0.76	1.08	1.98	1.77	2.25	1.05			1.86	9.33	1.79	5.88	0.90	9.09	1.23	1.30	1.96	1.83	1.29	0.70	2.83	3.25	1.56	1.37	1.01	6.39
P17	1.55	4.51	2.23	1.42	1.76	2.10	1.83	1.78	4.69	8.26			3.16	7.65				6.50	2.34		1.60		2.20	1.82	2.57	2.69	3.15	1.10	1.14	5.53
P18	1.57	4.33	2.17	1.49	1.74	2.23	1.94	1.80	4.01	7.95	1.30	9.24	3.48	7.82	4.05	4.76	1.70	6.54	2.46	2.73	1.59	1.60	2.29	1.78	2.40	2.53	2.97	1.09	1.28	
P19	1.90	4.80	2.39	1.47	1.89	2.19	1.86	1.81	3.08	7.47	1.39	8.89	3.34	8.11	4.46	4.88							1.65	1.66	2.32	2.59	2.11	1.01	1.36	5.83

Table 16

Peptide	142a	142b	142c	142h	145a	145b	145c	145h	146a	146b	146c	146h	sum_a	sum_b	sum_c	sum_h
VQE	-2.59	-2.64	-1.54	-0.06	1.75	0.50	-1.44	0.58	3.11	0.26	-0.11	0.1550	2.27	-1.88	-3.09	0.676
DYD	3.98	0.93	1.93	-0.197	-2.54	2.44	0.43	0.084	3.98	0.93	1.93	-0.197	5.42	4.3	4.29	-0.31
GRA	2.05	-4.06	0.36	-0.48	3.52	2.50	-3.5	0.93	0.24	-2.32	0.6	0.9990	5.81	-3.88	-2.54	1.449
GSQ	2.05	-4.06	0.36	-0.48	2.39	-1.07	1.15	0.16	1.75	0.5	-1.44	0.5770	6.19	-4.63	0.07	0.254
FGR	-4.22	1.94	1.06	0.08	2.05	-4.06	0.36	-0.48	3.52	2.5	-3.5	0.9280	1.35	0.38	-2.08	0.534
QDF	1.75	0.50	-1.44	0.58	3.98	0.93	1.93	-0.20	-4.22	1.94	1.06	0.0840	1.51	3.37	1.55	0.464
RDG	3.52	2.50	-3.50	0.93	3.98	0.93	1.93	-0.20	2.05	-4.06	0.36	-0.4780	9.55	-0.63	-1.21	0.253
DRK	3.98	0.93	1.93	-0.20	3.52	2.50	-3.5	0.93	2.29	0.89	-2.49	0.7170	9.79	4.32	-4.06	1.448
DSA	3.98	0.93	1.93	-0.20	2.39	-1.07	1.15	0.16	0.24	-2.32	0.6	0.9990	6.61	-2.46	3.68	0.957
NES	3.05	1.62	1.04	0.014	3.11	0.26	-0.11	0.155	2.39	-1.07	1.15	0.155	8.55	0.81	2.08	0.324
SEA	2.39	-1.07	1.15	0.155	3.11	0.26	-0.11	0.155	0.24	-2.32	0.6	0.999	5.74	-3.13	1.64	1.309
SAS	2.39	-1.07	1.15	0.155	0.24	-2.32	0.6	0.999	2.39	-1.07	1.15	0.155	5.02	-4.46	2.9	1.309

Table 17

DMka	=	Nakd	=	DMkd	=
-2.520	+	2.613	+	31.614	+
-0.129	*[142h] +	4.930	*[142h] +	-3.168	*[142h] +
0.086	*[145h] +	-0.852	*[145h] +	3.161	*[145h] +
-0.009	*[142b] +	-0.305	*[142b] +	-1.531	*[142b] +
0.112	*[142c] +	-0.748	*[142c] +	-0.406	*[142c] +
0.030	*[145a] +	1.444	*[145a] +	-1.090	*[145a] +
-0.002	*[145c] +	0.773	*[145c] +	-0.258	*[145c] +
0.086	*[146c] +	0.811	*[146c] +	1.700	*[146c] +
0.097	*[sum_c] +	1.094	*[sum_c] +	0.251	*[sum_c] +
-0.046	*[142c*142c] +	-0.235	*[142c*142c] +	-0.263	*[142c*142c] +
-0.028	*[sum_c*sum_c]	-0.841	*[sum_c*sum_c]	-0.059	*[sum_c*sum_c]

Table 18

Amino acid	a	b	c	h
DRK	+	+	-	+
DYD	-	+	0	+
DSA	+	0	0	0
DRK	+	0	-	+
DYD	+	0	+	-
DSA	0	-	0	+

EXAMPLE 6

This example illustrates a representative method for discriminating between human myoglobin and sheep myoglobin by use of a multi-buffer perturbation approach in accordance with the present invention. Specifically, the monoclonal antibodies 8e11 and 22:3 raised against human myoglobin bind to different epitopes. Thus, the two antibodies can be used to monitor presence of human myoglobin. The antibodies cross reacts with sheep myoglobin. 8e11 has similar binding characteristics toward both species while as 22:3 has lower affinity to sheep myoglobin than to human myoglobin.

Suppose that the two antibodies were used for Quality Control purposes. In that case, the goal is to have clearly different signals from the similar molecules human myoglobin and sheep myoglobin. In order to achieve such a method, biomolecular interaction analysis in a plurality of buffers was performed. The interaction between 8e11 and both myoglobins were only slightly perturbed by the buffer additives. For the 22:3 antibody, the interaction with sheep myoglobin was found to be more pH sensitive that the interaction with human myoglobin. The concentration of urea and NaCl also perturbed the interaction, but not as clear as pH. Four different buffers were pH, urea and NaCl were varied were chosen. By analyzing not only the binding rates for all buffers, but also the sensitivity pattern, a more sensitive assay was developed.

MATERIALS AND METHODS

Buffer-Kinetic relationships

Approximately 700RU monoclonal antibodies 22:3 (Biacore) and 8e11 (Biacore) was immobilized on Sensor Chip CM5 using standard amine coupling. Human myoglobin (Sigma) and sheep myoglobin (Biacore) were diluted in HBS-EP buffer (10 mM HEPES, 150 mM NaCl, 0.005% Surfactant P20, 3.4 mM EDTA, pH7.4) and in p-buffers (table 2). The myoglobins were injected for 120 s at flow 30µl/min using the TRIINJECT command in three concentrations ranging from 55nM to 588nM diluted in the designed set of P-buffers (Table 19). Kinetic parameters were calculated in BIAevaluation 3.0. For each peptide, a buffer-kinetics relationship (BKR) was calculated in MODDE 4.0 (Umetrics AB, Umeå, Sweden) (data not shown).

Table 19

Name	NaCl	KSCN	DMSO	pH	EDTA	urea
P1	150	4	30	7	3	40
P2	150	4	300	7	23	400
P3	150	22	30	7	23	400
P4	150	22	300	7	3	40
P5	550	4	30	7	23	40
P6	550	4	300	7	3	400
P7	550	22	30	7	3	400
P8	550	22	300	7	23	40
P9	150	4	30	7.8	3	400
P10	150	4	300	7.8	23	40
P11	150	22	30	7.8	23	40
P12	150	22	300	7.8	3	400
P13	550	4	30	7.8	23	400
P14	550	4	300	7.8	3	40
P15	550	22	30	7.8	3	40
P16	550	22	300	7.8	23	400
P17	350	13	165	7.4	13	220
P18	350	13	165	7.4	13	220
P19	350	13	165	7.4	13	220

Single concentration - four buffer experiments

Human myoglobin and sheep myoglobin was diluted in buffers P1, P7, P9, P15 and HBS-EP to 544nM (human) and 588nM (sheep). The myoglobin solutions were injected for 100s at flow 30 µl/min using the TRIINJECT command. The solutions were in most cases injected in duplicate, and the experiment was repeated three times using two instruments. The sensor chips were in two cases newly prepared chips and in one case a sensor chip that had been regenerated approximately 150 times prior to this experiment.

Discussion of Results

The buffer kinetics relationship showed that the interaction between antibody 8e11 and both myoglobins were very stable to perturbations of the chemical environment. The binding characteristics were comparable for both myoglobins (Figures 31a-b). For 22:3, sheep myoglobin dissociated faster than human myoglobin (Figures 32a-b). The interaction with sheep myoglobin was also more sensitive to

variations in buffer composition than human myoglobin. The dominating factors were pH, urea concentration and NaCl concentration. To clearly discriminate between the two myoglobins, the interaction was studied in four buffers. The four buffers were different with regards to pH, urea and NaCl and were chosen among the P-buffer set (P1, P7, P9, P15). One concentration was injected and two points in the dissociation phase was used for analysis. Results from the experiments are available presented in Table 20.

Table 20

		Experiment 1 new sensor chip			Experiment 2 new sensor chip			Experiment 3 old sensor chip			Error estimation of d1 and d2
	buffer	d1 RU	d2 RU	d1-d2 RU	d1 RU	d2 RU	d1-d2 RU	d1 RU	d2 RU	d1-d2 RU	RU
Mab 8ell	P1	93.1	92.4	0.7	70.5	70.1	0.4	82.7	82.5	0.02	0.86
Human	P15	70.1	69.6	0.5	57.9	57.1	0.8	67.3	68.2	-0.8	1.54
	P7	72.8	71.4	1.4	55.8	53.6	2.3	70.0	71.3	-1.3	5.80
	P9	69.1	67.4	1.7	58.7	57.7	0.9	64.3	64.3	-0.1	3.61
	HBS	81.6	80.1	1.5	60.8	59.4	1.4	75.2	75.4	-0.1	1.81
Mab 8ell	P1	82.5	81.6	1.0	65.5	64.4	1.1	74.9	74.7	0.3	0.76
Sheep	P15	62.1	60.8	1.3	51.6	50.6	1.0	57.4	58	-0.6	0.68
	P7	64.0	62.1	1.9	49.5	47.7	1.8	58.7	59.2	-0.5	0.82
	P9	58.6	57.4	1.2	48.8	47.9	0.9	53.3	53.7	-0.4	0.37
	HBS	73.9	72.0	1.8	60.6	59.6	1.0	67.4	67.5	-0.1	2.76
Mab 22:3	P1	50.7	47.4	3.3	72.4	67.9	4.5	78.0	74.1	3.8	1.42
Human	P15	50.5	48.3	2.2	79.9	77.1	2.8	82.7	81	1.7	0.91
	P7	49.9	45.4	4.5	75.7	69.2	6.5	80.1	76.7	3.4	5.35
	P9	45.1	42.6	2.5	74.3	70.4	4.0	72.0	69.4	2.6	1.58
	HBS	49.3	46.3	3.0	69.6	65.5	4.1	75.8	73.3	2.5	0.73
Mab 22:3	P1	53.4	31.7	21.7	73.7	40.0	33.7	77.2	44.5	32.7	1.68
Sheep	P15	58.4	46.9	11.5	81.8	66.8	15.0	90.0	74.5	15.5	0.47
	P7	47.5	21.8	25.7	66.4	27.9	38.5	70.6	32.0	38.6	1.18
	P9	50.7	35.2	15.5	74.9	53.9	21.0	79.8	57.7	22.1	1.24
	HBS	57.4	40.9	16.5	79.6	58.6	21.0	83.6	61.8	21.8	2.77

The results show that it is possible to discriminate human myoglobin from sheep myoglobin on a sensorgram basis if the dissociation for the interaction myoglobin - mab 22:3 calculated; however, the discrimination becomes clearer if several buffers are used since the interaction of sheep myoglobin - mab 22:3 is highly sensitive to chemical additives in contrast to the stable interaction of human myoglobin - mab 22:3.

The results were converted to % of HBS-levels to eliminate influences of absolute signal levels (due to different immobilization levels, for example) (Table 21). By plotting D1 vs. D1-D2 for all four buffers, a picture of the sensitivity is obtained (Figures 33a-33c). By comparing the Figures, sheep and human myoglobin can reliably be differed from each other. The method is also reliable for frequently used sensor chips.

Table 21

		Experiment 1 new sensor chip			Experiment 2 new sensor chip			Experiment 3 old sensor chip		
	buffer	d1 % of hbs	d2 % of hbs	d1-d2 % of d1	d1 % of hbs	d2 % of hbs	d1-d2 % of d1	d1 % of hbs	d2 % of hbs	d1-d2 % of d1
Mab 8ell Human	P1	114	115	1	116	118	1	110	109	0
	P15	86	87	1	95	96	1	89	90	-1
	P7	89	89	2	92	90	4	93.0	95	-2
	P9	85	84	2	96	97	2	85	85	0
Mab 8ell Sheep	P1	112	113	1.0	108	108	2	111	111	0
	P15	84	84	2	85	85	2	85	86	-1
	P7	87.0	86	3	82	80	4	87	88	-1
	P9	79	80	2	81	80	2	79	80	-1
Mab 22:3 Human	P1	103	102	7	104	104	6	103	101	5
	P15	103	104	4	115	118	4	109	111	2
	P7	101	98	9	109	106	9	106	105	4
	P9	91	92	5	107	107	5	95	95	4
Mab 22:3 Sheep	P1	93	78	41	93	68	46	92	72	42
	P15	102	115	20	103	114	18	108	121	17
	P7	83	53	54	83	48	58	84	52	55
	P9	88	86	31	94	92	28	95	93	28

Conclusion

It is possible to discriminate between sheep myoglobin and human myoglobin by comparing dissociation, but when the interaction is studied in a plurality of buffers, the difference between the two myoglobins becomes larger. Thus, the multi-buffer method increases the reliability when differing the myoglobins from each other. In a quality control situation, methods and clear signals differing similar molecules from each other is needed. The multi-buffer method used in this report fulfills that requirement when differing the two myoglobins from each other.

From the foregoing, it will be understood that, although specific embodiments of this invention have been described herein for purposes of illustration, various modifications may be made without departing from the spirit and scope of the invention. Accordingly, the invention is not limited except by the appended claims.

CLAIMS

1. A method for selecting an optimized regeneration solution for the regeneration of a biosensor surface having a surface-bound ligand and an analyte associated with the ligand, comprising:

(a) sequentially contacting the biosensor surface with each of a plurality of first regeneration cocktails, wherein each of said first regeneration cocktails is an aqueous solution comprising at least one acidic, basic, ionic, organic, detergent or chelating stock solution, and wherein at least one of said first regeneration cocktails comprises a mixture of at least two of said stock solutions;

(b) measuring the regeneration effect for each of said plurality of first regeneration cocktails to determine which of said plurality of first regeneration cocktails have the highest measured regeneration effect;

(c) selecting at least two different stock solutions present in said plurality of first regeneration cocktails having the highest measured regeneration effect;

(d) combining said at least two different stock solutions in various ratios to generate a plurality of second regeneration cocktails;

(e) sequentially contacting the biosenor surface with each of said plurality of second regeneration cocktails; and

(f) determining the regeneration effect of each of said plurality of second regeneration cocktails and therefrom identifying a second regeneration cocktail as the optimized regeneration solution.

2. The method of claim 1 wherein, during the step of sequentially contacting the biosensor surface with each of said plurality of first regeneration cocktails, the biosensor surface is contacted with an additional quantity of analyte to associate said additional quantity of analyte with the surface-bound ligand.

3. The method of claim 1 wherein the ligand and analyte are a binding pair selected from antibody-antigen, hormone-hormone receptor, polynucleotide-complementary polynucleotide, avidin/streptavidin-biotin, enzyme-enzyme substrate or inhibitor, lectins-specific carboxyhydrate, lipids-lipid binding proteins or membrane-associated proteins, polynucleotides-polynucleotide binding proteins, receptor-transmitter, drug-target, protein-protein, protein-polynucleotide, DNA-DNA, and DNA-RNA.

4. The method of claims 1 wherein the ligand is an antibody and the analyte is an antigen.

5. The method of claim 1 wherein said mixture of at least two of said stock solutions is selected from a mixture of basic and acidic stock solutions, basic and detergent stock solutions, basic and chelating stock solutions, acidic and ionic stock solutions, acidic and nonpolar stock solutions, acid and detergent stock solutions, acid and chelating stock solutions, ionic and detergent stock solutions, ionic and chelating stock solutions, detergent and nonpolar stock solutions, detergent and chelating stock solutions, and nonpolar and chelating stock solutions.

6. The method of claim 1 wherein said plurality of first regeneration cocktails are aqueous solutions containing an acidic stock solution, a basic stock solution, an ionic stock solution, a nonpolar stock solution, a detergent stock solution, a chelating stock solution. a mixture of basic and acidic stock solutions, a mixture of basic and detergent stock solutions, a mixture of basic and chelating stock solutions, a mixture of acidic and ionic stock solutions, a mixture of acidic and nonpolar stock solutions, a mixture of acid and detergent stock solutions, a mixture of acid and chelating stock solutions, a mixture of ionic and detergent stock solutions, a mixture of ionic and chelating stock solutions, a mixture of detergent and nonpolar stock solutions, a mixture of detergent and chelating stock solutions, and a mixture of nonpolar and chelating stock solutions.

7. The method of claim 1 wherein said acidic stock solution comprises a mixture of acids having dispersed pKs ranging from about 2 up to 7.

8. The method of claim 7 wherein said acidic stock solution has a pH ranging from 1 up to 7.

9. The method of claim 7 wherein said acidic stock solution is a mixture of acids comprising at least two acids selected from arsenic acid, arsenious acid, o-boric acid, carbonic acid, chromic acid, germanic acid, hyrocyanic acid, hydrofluoric acid, hydrogen sulfide, hydrogen peroxide, hypobromus acid, hypochlorous acid, hypoiodous acid, iodic acid, nitrous acid, periodic acid, o-phosphoric acid, phosphorous acid, pyrophosphoric acid, selenic acid, selenious acid, m-silic acid, o-silic acid, sulfuric acid, sulfurous acid, telluric acid, tellurous acid, tetraboric acid, acetic acid, acetoacetic acid, acrylic acid, adipamic acid,

adipic acid, d-alanine, allantoin, alloxanic acid, glycine, o-aminobenzoic acid, m-aminobenzoic acid, p-aminobenzoic acid, o-aminobenzsulfonic acid, m-aminobenzsulfonic acid, p-aminobenzsulfonic acid, ainsic acid, o-beta-anisylpropionic acid, m-beta-anisylpropionic acid, p-beta-anisylpropionic acid, ascorbic acid, DL-aspartic acid, barbituric acid, benzoic acid, benzosoulfonic acid, bromoacetic acid, o-bromobenzoic acid, m-bromobenzoic acid, n-butyric acid, iso-butyric acid, cyclopropane-1:1-dicarboxylic acid, DL-cystein, L-cystein, dichloroacetic acid, dichloroacetylacetic acid, 2,3-dichlorophenol, 2,2-dihydroxybenzoic, 2,5-dihydroxybenzoic, 3,4-dihydroxybenzoic, 3,5-dihydroxybenzoic, dihydroxymalic acid, dihydroxytartaric acid, dimethylglycine, dimethylmalic acid, dimethylmalonic acid, dinicotinic acid, 2,4-dinitrophenol, 3,6-dinitrophenol, diphenylacetic acid, ethylbenzoic acid, ethylphenylacetic acid, fluorobenzoic acid, formic acid, fumaric acid, furancarboxylic acid, furoic acid, cacodylic acid, n-caproic acid, iso-caproic acid, chloroacetic acid, o-chlorobenzoic acid, m-chlorobenzoic acid, p-chlorobenzoic acid, o-chlorobutyric acid, m-chlorobutyric acid, p-chlorobutyric acid, o-chloroinnamic acid ,m-chloroinnamic acid, p-chloroinnamic acid, o-chlorophenoxyacetic acid, m-chlorophenoxyacetic acid, p-chlorophenoxyacetic acid, o-chlorophenylacetic acid, m-chlorophenylacetic acid, p-chlorophenylacetic acid, beta-(o-chlorophenyl) propionic acid, beta-(m-chlorophenyl) propionic acid, beta-(p-chlorophenyl) propionic acid, alfa-chloropropinic acid, beta-chloropropionic acid, cis-cinnamic acid, trans-cinnamic acid, citric acid, o-cresol, m-cresol, p-cresol, trans-crotonic acid, cyanoacetic acid, gamma-cyanobutyric acid, o-cyanophenoxyacetic acid, p-cyanophenoxyacetic acid. cyanopropionic acid, cycloheaxane-1:1-dicarboxylic acid, gallic acid, glutamaric acid, glutaric acid, glycerol, glycine, glycol, glycolic acid, heptanoic acid, hexahydrobenzoic acid, hexanoic acid, hippuric acid, histidine, hydroquinone, o-hydroxybenzoic acid, m-hydroxybenzoic acid, p-hydroxybenzoic acid, beta-hydroxybutyric acid, gamma-hydroxybutyric acid, beta-hydroxypropionic acid, gamma-hydroxyquinoline, iodoacetic acid, o-iodobenzoic acid, m-iodobenzoic acid, itaconic acid, lactic acid, lutidinic acid, lysine, maleic acid, malic acid, malonic acid, DL-mandelic acid, mesaconic acid, mesitylenic acid, methyl-o-aminobenzoic acid, methyl-m-aminobenzoic acid, methyl-p-aminobenzoic acid, o-methylcinnamic acid, m-methylcinnamic acid, p-methylcinnamic acid, beta-methylglutaric acid, n-methylglycine, methylmalonic acid, methylsuccinic acid, o-monochlorophenol, m-monochlorophenol, p-monochlorophenol, o-phthalic acid, m-phthalic acid, p-phthalic acid, picric acid, pimelic acid, propionic acid, iso-propylbenzoic acid, 2-pyridinecarboxylic acid, 3-pyridinecarboxylic acid, 4-pyridinecarboxylic acid, pyrocatcchol, quinolinic acid, Resorcinol, Saccarin, suberic acid, succinic acid, sulfanilic acid, naphtalenesulfonic acid, alfa-naphthoic acid, beta-naphthoic acid, alfa-napthol, beta-napthol, nitrobenzene, o-nitrobenzoic acid,

m-nitrobenzoic acid, p-nitrobenzoic acid, o-nitrophenol, m-nitrophenol, p-nitrophenol, o-nitrophenylacetic acid, m-nitrophenylacetic acid, p-nitrophenylacetic acid, o-beta nitrophenylpropionic acid, p-beta nitrophenylpropionic acid, nonanic acid, octanic acid, oxalic acid, phenol, phenylacetic acid, o-phenylbenzoic acid, gamma-phenylbutyric acid, alfa-phenylpropionic acid, beta-phenylpropionic acid, alfa-tataric acid, alfa-tartaric acid, meso-tartaric acid, theobromine, terephtalic acid, thioacetic acid, thiophenecarboxylic acid, o-toluic acid, m-toluic acid, p-toluic acid, trichloroacetic acid, trichlorophenol, 2,4,6-trihydroxybenzoic acid, trimethylacetic acid, 2,4,6-trinitrophenol, tryptophan, tyrosine, uric acid, n-valeric acid, iso-valeric acid, veronal, vinylacetic acid, and xanthine.

10. The method of claim 7 wherein said acidic stock solution is a mixture of acids comprising oxalic, phosphoric, formic and malonic acid.

11. The method of claim 1 wherein said basic stock solution comprises a mixture of bases having dispersed pKs ranging from in excess of 7 up to about 12.

12. The method of claim 11 wherein said basic stock solution is a mixture of bases comprising at least two bases selected from acetamide, acridin, alfa-alanin, gylcyl alanin, methoxy (DL) - alanin, phenyl alanin, allothreonin, n-amylamine, aniline, n-allyl aniline, 4-(p-aminobenzoyl) aniline, 4-benzyl aniline, 2-bromo aniline, 3-bromo aniline, 4-bromo aniline, 4-bromo-N,N,dimethyl aniline, o-chloro aniline, m- chloro aniline, p- chloro aniline, 3-bromo-N,N,dimethyl aniline, 4-bromo-N,N,dimethyl aniline, 3,5-dibromo Aniline, 2,4-dichloro aniline, N,N-diethyl aniline, N,N-dimethyl-3-nitro aniline, N-ethyl aniline, 2-fluoro aniline, 3- fluoro aniline, 4- fluoro aniline, 2-iodo aniline, N-methyl aniline, N-methylthio aniline, 3- nitro aniline, 4- nitro aniline, 2-sulfonic acid aniline, 3-sulfonic acid aniline, 4-sulfonic acid aniline, brucine, 1-amino-3-methyl-butane, 2-amino-4-methyl-butane, 1,4-diamino-butane, n-butylamine, t-butylamine, 4-amino butyric acid, lycyl-2-amino n-butyric acid, cacodylic acid, beta-chlortriethylammonium, cinnoline, codeine, n-butyl-cyclohexanamin, cyclohexanamin, cystin, n-decylamine, diethylamine, diisobutylamine, diisopropylamine, dimethylamine, n-diphenylamine, n-dodecaneamine, d-ephedrine, l-ephedrine, 1-amino-3-metoxy-ethane, 2-amino ethanole, o-anisidine, m-anisidine, p-anisidine, arginin, asparagin, glycylasparagin, DL-aspartic acid, azetidin, aziridine, 4-aminoazo benzene, 2-aminoethyl benzene, 4-dimethylaminoazo benzene, benzidine, benzimidazole, 2-ethyl benzimidazole, 2-methyl benzimidazole, 2-phenyl benzimidazole, 2-amino benzoic acid, 4-amino benzoic acid, benzylamine, betaine, 2-amino biphenyl, trans-bornylamine, ehtylamine, ethylenediamine, l-Glutamic acid, alfa-monoethyl

glutamic acid, l-glutamine, l-glutathione, glycine, n-acetyl glycine, dimethyl glycine, glycyl glycine, glycylglycyl glycine, leucyl glycine, methyl glycine, phenyl glycine, N,n-propyl glycine, tetraglycyl glycine, glycylserine, hexadecanamine, 1-amino heptan, 2-amino-heptan, 2-metylamino heptan, hexadecanamine, hexamethylenediamine, 6-amino hexanoic acid, n-hexylamine, dl-histidine, beta-analyl histidine, imidazol, 2,4-dimethyl imidazol, 1-methyl imidazol, 1-amino indane, 2-amino isobutyric acid, isoleucin, isoquinolin, 1-amino isoquinolin, 7-hydroxy isoquinolin, L-leucin, glycyl leucin, methionin, metylamine, morphine, morpholine, 1-amino-6-hydroxy naphtalene, dimethylamino naphtalene, alfa-naphthylamine, beta-napthylamine, piperazine, 2,5,dimetyl(trans)piperazine, piperidine, 3-acetyl piperidine, 1-n-butyl piperidine, 1,2-dimethyl piperidine, 1-ethyl piperidine, 1-methyl piperidine, 2,2,6,6,tetramethyl piperidine, 2,2,4-trimethyl piperidine, proline, hydroxyproline, 1-amino-2,2-dimethylpropane, 1,2-diaminopropane, 1,3-diaminopropane, 1,2,3-triaminopropane, 3-amino propanoic acid, propylamine, pteridine, 2-amino-4-hydroxy pteridine, 2-amino-4,6-dihydroxy pteridine, 6-chloro pteridine, 6-hydroxy-4-methyl pteridine, purine, n-methyl alfa-naphtylamine, cis-neobornylamine, nicotine, n-nonylamine, norleucine, octadecanamine, octylamine, ornithine, papaverine, 3-amino pentane, 3-amino-3-methyl pentane, n-pentadecylamine, 5-amino pentanoic acid, perimidine, phenanthridine, 1,10-phenanthroline, o-phenetidine, m-phenetidine, p-phenetidine, alfa-picoline, beta-picoline, gamma-picoline, pilocarpine, 6-amino purin, 2-dimethylaminopurine, 8-hydroxy purin, pyrazin, 2-methylpyrazine, methylaminopyrazine, pyrdazine, 2-aminopyrimidine, 2-amino-4,6-dimethylpyrimidine, 2-amino-5-nitro pyrimidine, pyridine, 2-amino pyridine, 4-amino pyridine, 2-benzyl pyridine, 3-bromo pyridine, 3-chloro pyridine, 2,5-diamino pyridine, 2,3-dimethyl pyridine, 2,4-dimethyl pyridine, 2-ethyl pyridine, 2-formyl pyridine, a-hydroxy pyridine, 4-hydroxy pyridine, methoxy pyridine, 4-methylamino pyridine, 2,4,6,trimetyl pyridine, pyrrolidine, 1,2-dimethyl pyrrolidine, n-methyl pyrrolidine, quinazoline, 5-hydroxy quinazoline, quinine, quinoline, 3-amino quinoline, 3-bromo quinoline, 8-carboxy quinoline, 3-hydroxy quinoline, 8-hydroxy quinoline, 8-hydroxy-5-sulfo quinoline, 6-methoxy quinoline, 2-methyl quinoline, 4-methyl quinoline, 5-methyl quinoline, quinoxaline, serine, strychnine, taurine; tetradecaneamin, thiazole, 2-aminothiazole, threonine, o-toluidine, m-toluidine, p-toluidine, 2,4,6-triamino-1,3,5-triazine, tridecanamine, triethylamine, trimethylamine, tryptophan, tyrosine, urea, valine, ammonium hydroxide, arsenous oxide, beryllium hydroxide, calcium hydroxide, deuteroammonium hydroxide, hydrazine, hydroxylamine, lead hydroxide, silver hydroxide, and zinc hydroxide.

13. The method of claim 11 wherein said basic stock solution is a mixture of bases comprising ethanolamine, sodium phosphate, piperazin and glycine.

14. The method of claim 1 wherein said ionic stock solution comprises a mixture of at least two ions selected from PO_4^{3-}, SO_4^{2-}, CH_3COO^-, Br^-, NO_3^-, NO_2^-, ClO_4^-, Cl^-, F^-, I^-, CF_3COO^-, SCN^-, Cl_3COO^-, CCl_3COO^-, $(CH_3)_4N^+$, NH_4^+, Rb^+, K^+, Na^+, Cs^+, Li^+, Mg^{2+}, Ca^{2+}, Sr^{2+}, and Ba^{2+}.

15. The method of claim 14 wherein said ionic stock solution comprises potassium thiocyanate, magnesium chloride, urea and guanidine HCl.

16. The method of claim 1 wherein said organic stock solution comprises a mixture of at least two organic solvents selected from DMSO, formamide, ethanol, acetonitrile, 1-butanol, acetone, methyl acetate, dichloroethane, chloroform, methyl alcohol, tetrahydrofuran, *n*-hexane, diisopropyl ether, ethyl acetate, ethyl alcohol, butanone, n-hexane, 2-propanol, 1,2-dichloroethane, flourobenzene, acetone, trichloroethylene, triethylamine, 1-propanol, butyronitrile, 2-butanol, nitromethane, dioxane, 2,2-dimethylpropanole, 3-pentanone, piperazine, 3-propanol, pyridin, 1-butanol, acetic acid, 2-metoxy ethanol, 3-methyl-1-butanol, chlorobenzene, acetic anhydride, dimethylformamide, methoxybenzene, methylbutylketone, bromobenzene, 1-hexanol, n-methyl formamide, aniline, iodobenzene, glycol, phenyl acetate, n-methyl formamide, benzyl alcohol, formamide, nitrophenol, diethyleneglycol, diphenylether, sulfolan, diethylether, methylene chloride, carbon disulfide, carbon tetrachloride, benzene, acetonitrile, toluene, dibutyl ether, dimethylbenzene, ortho-dichlorobenzene, and benzonitrile.

17. The method of claim 16 wherein said nonpolar stock solution comprises DMSO, formamide, ethanol, acetonitrile and 1-butanol.

18. The method of claim 1 wherein said detergent stock solution comprises a mixture of at least two detergent agents selected from anionic detergents, cationic detergents, zwitterionic detergents, and nonionic detergents.

19. The method of claim 18 wherein said detergent stock solution comprises CHAPS, Zwittergent 3-12, tween 80, tween 20 and triton X-100.

20. The method of claim 1 wherein said chelating stock solution comprises at least one chelating agent selected from EDTA, EGTA, NTA, DCYTA, GLEDTA, ETHEDTA, IDA and crown ethers.

21. The method of claim 20 wherein said chelating stock solution comprises EDTA.

22. The method of claim 1 wherein each of said stock solutions contains at least 20mM of each individual component present within said stock solution.

23. The method of claim 1 wherein each of said plurality of first regeneration cocktails contains a relative volume ratio of water to total stock solution in an amount ranging from 2:1 to 1:2

24. The method of claim 1 wherein the biosensor surface has a gold layer thereon which is capable of supporting surface plasmon resonance, and wherein the ligand is bound directly or indirectly to the gold layer.

25. The method of claim 4 wherein a dextran matrix is bound to the gold layer and the ligand bound to the dextan matrix.

26. The method of claim 1 wherein said at least two different stock solutions are two different stock solutions.

27. The method of claim 1 wherein said at least two different stock solutions are three different stock solutions.

28. The method of claim 1 wherein said at least two different stock solutions are four different stock solutions.

29. The method of claim 1, further comprising, after step (f):

(g) combining said at least two different stock solutions in different ratios than step (d) to generate a plurality of third regeneration cocktails;

(h) sequentially contacting the biosensor surface with each of said plurality of third regeneration cocktails; and

(i) determining the regeneration effect of each of said plurality of third regeneration cocktails and therefrom identifying a third regeneration cocktail as the optimized regeneration solution.

30. The method of claim 29 wherein steps (f) through (h) yields an optimized regeneration solution having a higher regeneration effect than the optimized regeneration solution identified in step (e).

31. The method of claim 1, further comprising:

(g) collecting the analyte for subsequent analysis.

32. A reagent kit comprising at least two different stock solutions for use in the method of claim 1 wherein said stock solutions are selected from an acidic stock solution, a basic stock solution, an ionic stock solution, an organic stock solution, a detergent stock solution and a chelating stock solution.

33. The reagent kit of claim 32 comprising at least three different stock solutions.

34. The reagent kit of claim 32 comprising four to five different stock solutions.

35. The reagent kit of claim 32 comprising six different stock solutions.

36. A computer system for selecting an optimized regeneration solution for the regeneration of a biosensor surface having a surface-bound ligand and an analyte associated with the ligand, comprising:

instructing a device to combine a series of stock solutions in various ratios to generate a plurality of first regeneration cocktails;

sequentially controlling the device to contact the biosensor surface with each of said plurality of first regeneration cocktails;

determining the regeneration effect of each of said first regeneration cocktails on the biosensor surface based on measurements received from the device;

selecting at least two different stock solutions having the highest regeneration effect;

instructing the device to combine a subset of said at least two different stock solutions in varying ratios to generate a plurality of second regeneration cocktails;

sequentially controlling the device to contact the biosenor surface with each of said second regeneration cocktails; and

determining the regeneration effect of each of said second regeneration cocktails based on measurements received from the device, and therefrom identifying a second regeneration cocktail as the optimized regeneration solution.

37. A computer-readable medium containing instructions for performing the method of claim 36.

38. A method for characterizing a ligand or analyte associated with a biosensor surface, comprising the steps of:

(a) sequentially contacting the biosensor surface having a surface-bound ligand with each of a plurality of characterization solutions;

(b) introducing the analyte into each of said plurality of characterization solutions so as to interact the analyte with the surface-bound ligand;

(c) measuring at least one of an association rate, surface-bound analyte concentration, dissociation rate, and regeneration effect of the analyte-ligand interaction for each of said plurality of characterization solutions; and

(d) characterizing the ligand or analyte associated with the biosensor surface based on at least one of the association rate, surface-bound analyte concentration, dissociation rate, and regeneration effect of the analyte-ligand interaction for each of said plurality of characterization solutions.

39. The method of claim 38, further comprising, after step (d):

(e) comparing the characterization of the ligand and/or analyte associated with the biosensor surface with a set of predetermined characterizations of other test molecules and thereby predicting the activity thereof.

40. The method of claim 39 wherein the set of predetermined characterizations of other test molecules includes a plurality of analogues of the ligand or analyte.

41. The method of claim 38 wherein each of said characterization solutions is an aqueous solution comprising at least one acidic, basic, ionic, organic, detergent or chelating solution.

42. The method of claim 41 wherein each aqueous solution is a single component solution.

43. The method of claim 41 wherein at least one of said characterization solutions comprises a mixture of at least two stock solutions.

44. The method of claim 41 wherein said plurality of characterization solutions are aqueous solutions selected from an acidic stock solution, a basic stock solution, an ionic stock solution, a nonpolar stock solution, a detergent stock solution, a chelating stock solution, a mixture of basic and acidic stock solutions, a mixture of basic and detergent stock solutions, a mixture of basic and chelating stock solutions, a mixture of acidic and ionic stock solutions, a mixture of acidic and nonpolar stock solutions, a mixture of acid and detergent stock solutions, a mixture of acid and chelating stock solutions, a mixture of ionic and detergent stock solutions, a mixture of ionic and chelating stock solutions, a mixture of detergent and nonpolar stock solutions, a mixture of detergent and chelating stock solutions, and a mixture of nonpolar and chelating stock solutions.

45. The method of claim 41, further comprising, after step (d):

(e) using a mathematical model wherein a structure of the analyte or ligand is given for predicting the activity of the analyte or ligand.

46. The method of claim 45 wherein the mathematical model uses pattern recognition algorithms.

47. The method of claim 41 wherein characterizing the ligand or analyte associated with the biosensor surface based on at least one of the association rate, surface-bound analyte concentration, dissociation rate, and regeneration effect of the analyte-ligand interaction for each of said plurality of characterization solutions comprises:

using a mathematical model having properties for determining a biospecific interaction effect of the analyte or ligand.

48. The method of claim 38 wherein the plurality of characterization solutions are at at least two different temperatures.

49. A computer memory containing a data structure useful for communicating chemical perturbation information associated with an analyte-ligand interaction, the data structure comprising one or more kinetic parameters, each kinetic parameter expressed in terms of a mathematical model that describes the relation between the kinetic parameter and the analyte-ligand interaction in a plurality of characterization solutions, such that the data structure may be used to communicate chemical perturbation information associated with the analyte-ligand interaction.

50. A generated data signal conveying a data structure useful for communicating chemical perturbation information associated with an analyte-ligand interaction, the data structure comprising one or more kinetic parameters, each kinetic parameter expressed in terms of a mathematical model that describes the relation between the kinetic parameter and the analyte-ligand interaction in a plurality of characterization solutions, such that the data structure may be used to communicate chemical perturbation information associated with the analyte-ligand interaction.

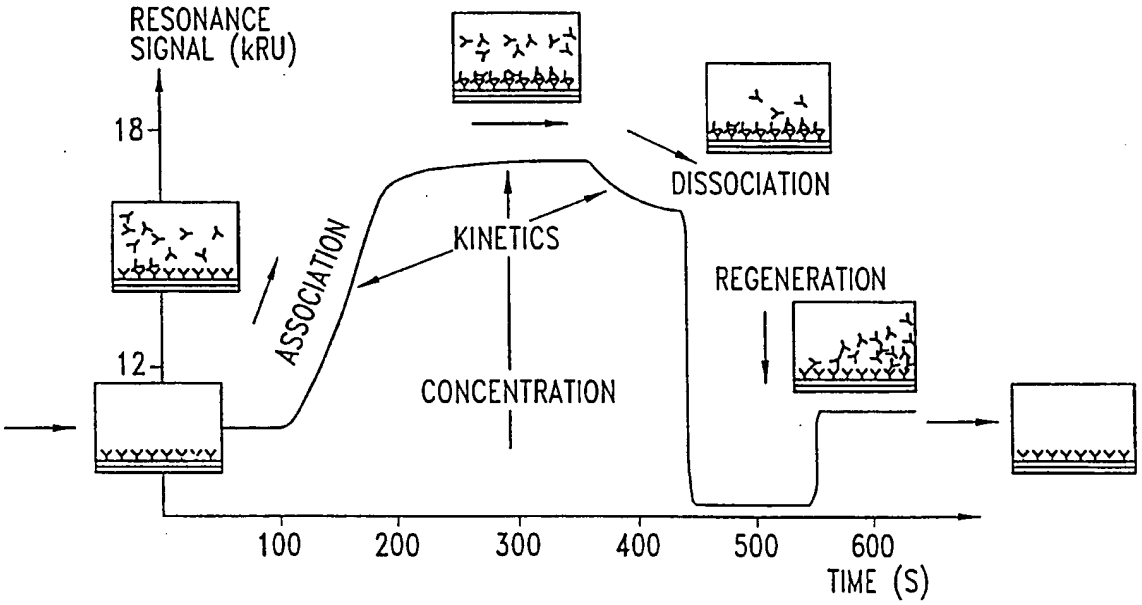
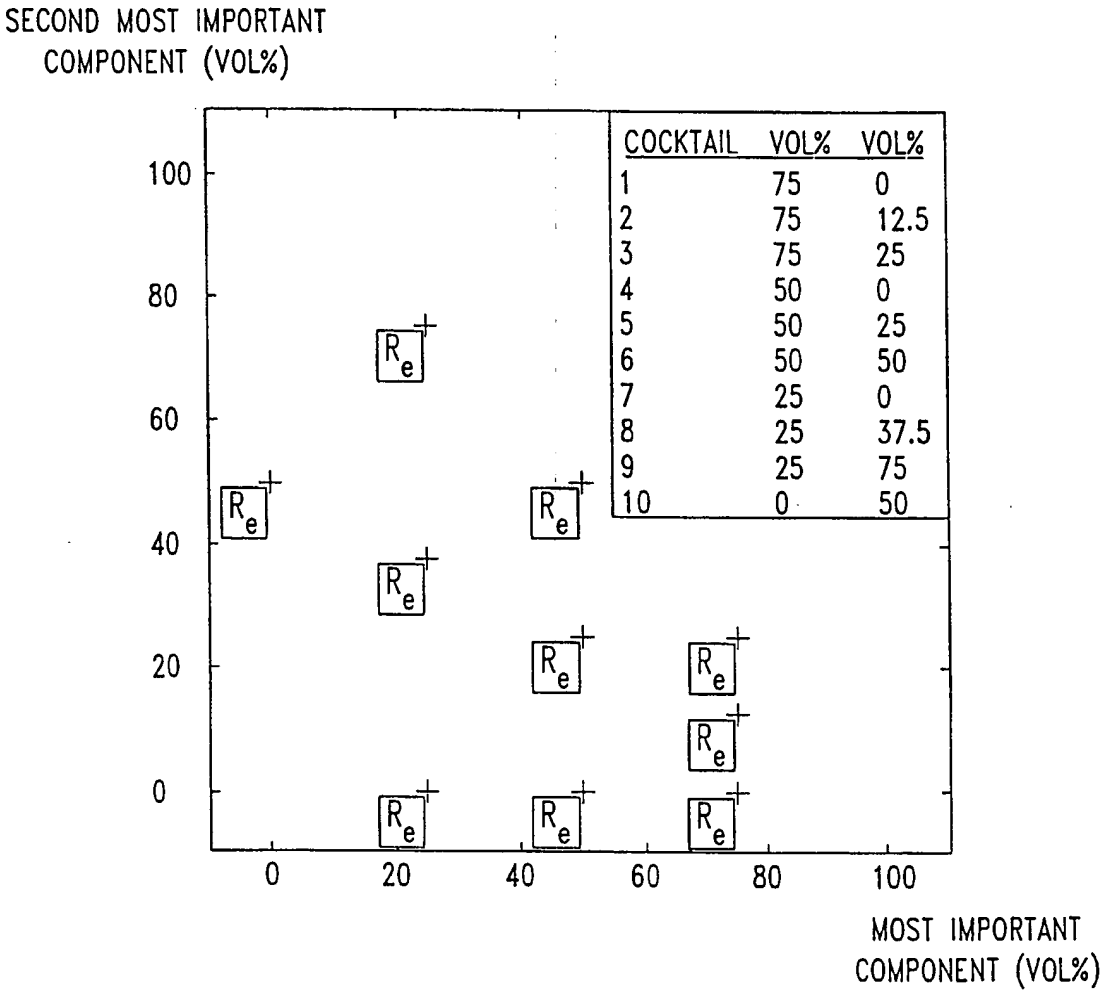


Fig. 1

(Prior Art)



COCKTAIL	VOL%	VOL%
1	75	0
2	75	12.5
3	75	25
4	50	0
5	50	25
6	50	50
7	25	0
8	25	37.5
9	25	75
10	0	50

Fig. 2

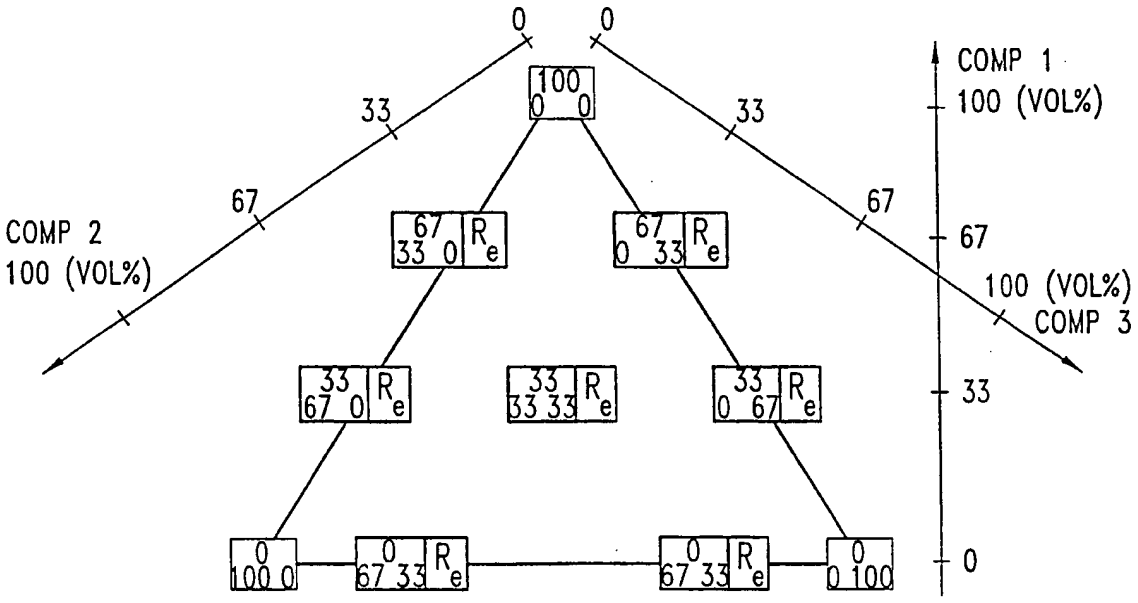

0
0
33
67
COMP 2
100 (VOL%)
33
67
COMP 1
100 (VOL%)
67
100 (VOL%)
COMP 3
33
0
100 0 0
67 33 0 Re
0 33 67 Re
33 67 0 Re
33 33 33 Re
33 0 67 Re
0 100 0
0 67 33 Re
0 67 33 Re
0 0 100

Fig. 3

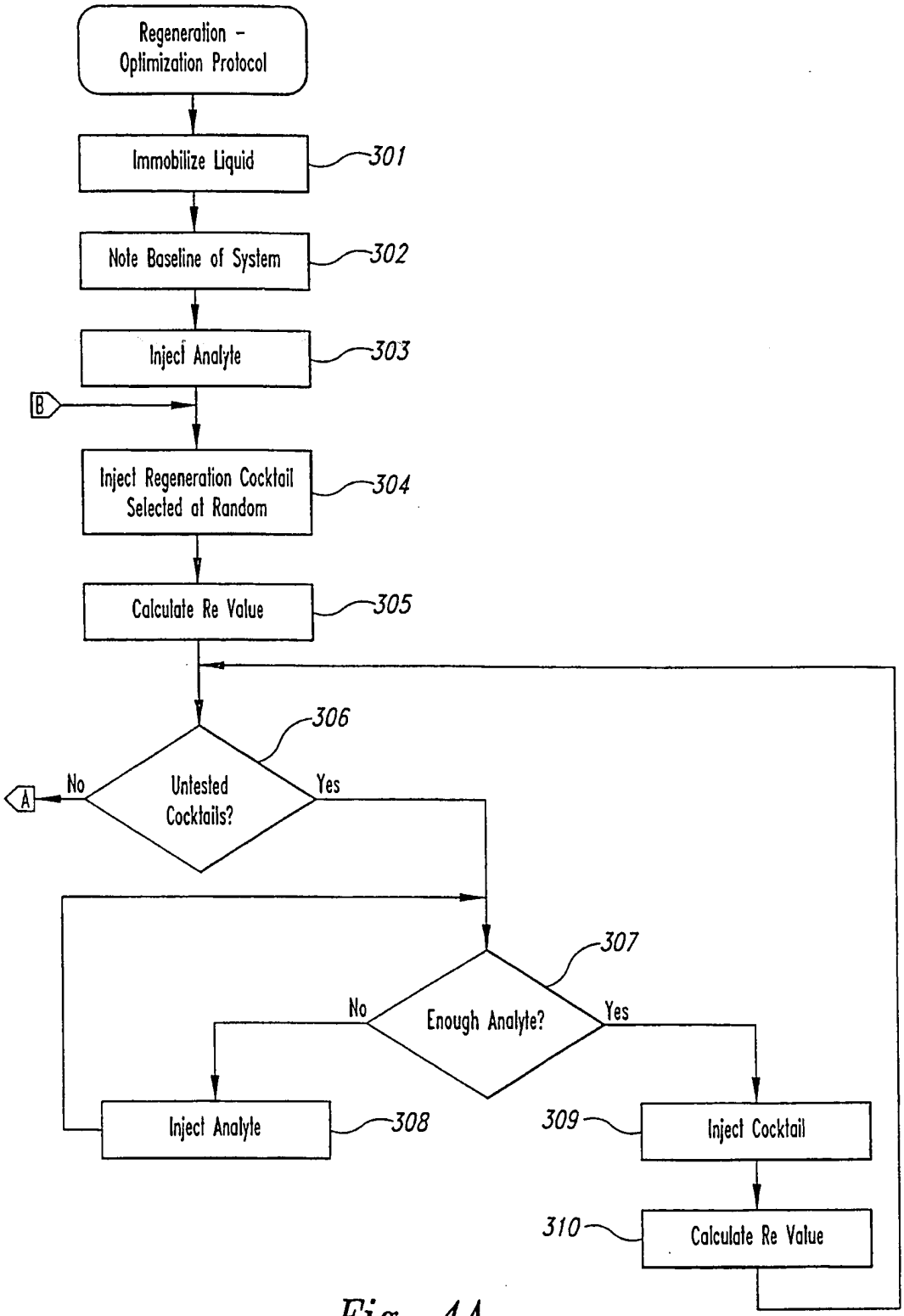

Regeneration - Optimization Protocol
Immobilize Liquid
301
Note Baseline of System
302
Inject Analyte
303
B
Inject Regeneration Cocktail Selected at Random
304
Calculate Re Value
305
306
Untested Cocktails?
No
Yes
A
307
Enough Analyte?
No
Yes
Inject Analyte
308
309
Inject Cocktail
310
Calculate Re Value

Fig. 4A

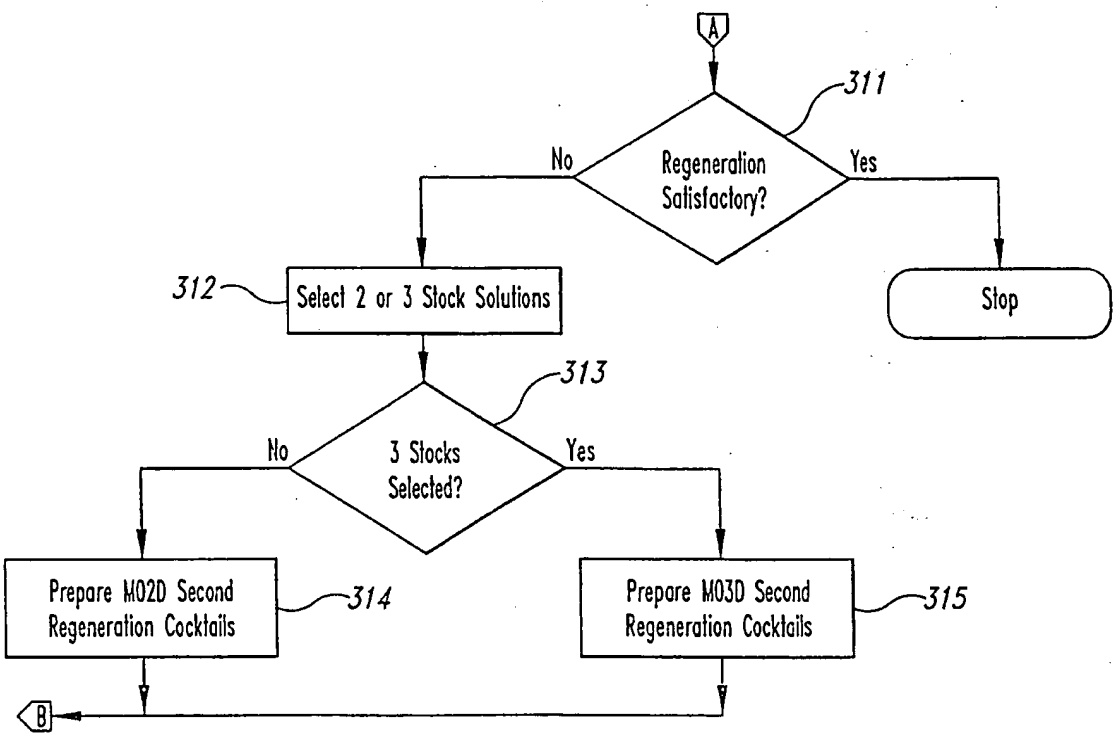

A
311
Regeneration Satisfactory?
No
Yes
312
Select 2 or 3 Stock Solutions
Stop
313
3 Stocks Selected?
No
Yes
Prepare M02D Second Regeneration Cocktails
314
Prepare M03D Second Regeneration Cocktails
315
B

Fig. 4B

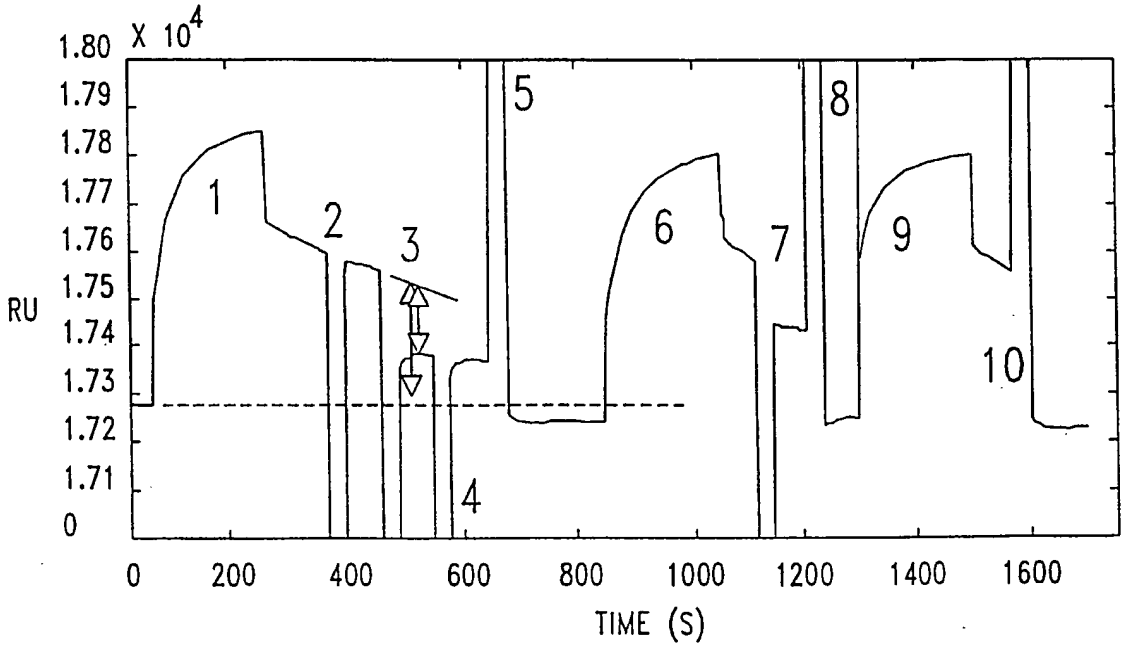

X 10^4
1.80
1.79
1.78
1.77
1.76
1.75
1.74
1.73
1.72
1.71
0
RU
1
2
3
4
5
6
7
8
9
10
0
200
400
600
800
1000
1200
1400
1600
TIME (S)

Fig. 5

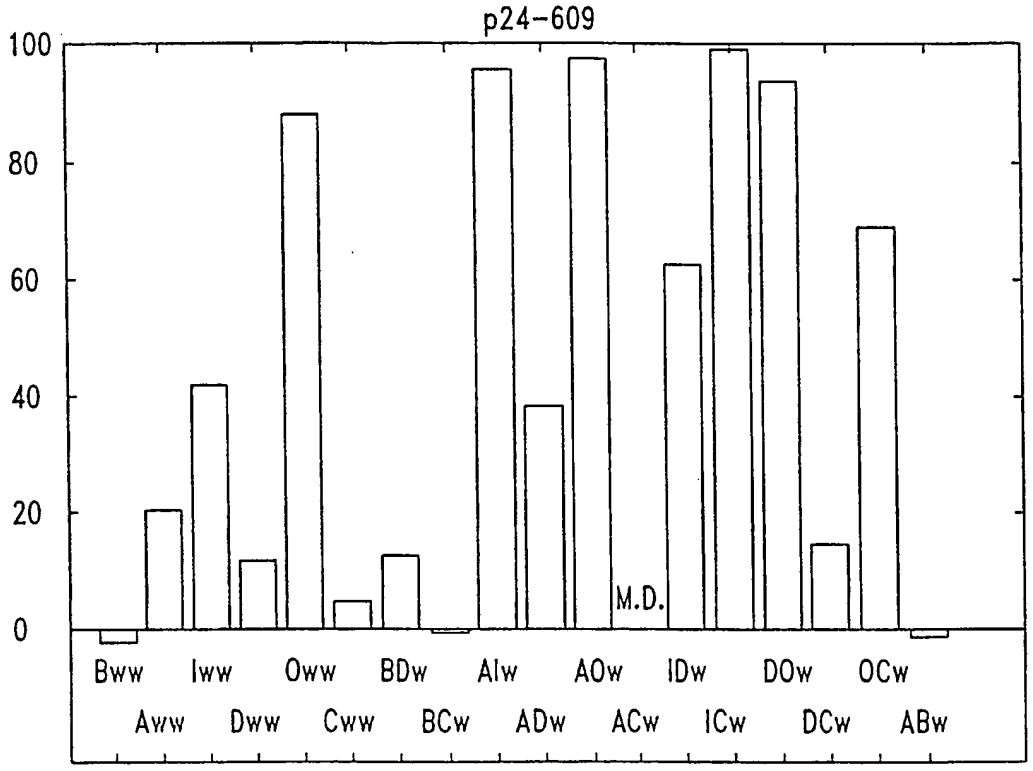

p24-609
100
80
60
40
20
0
M.D.
Bww
Iww
Oww
BDw
AIw
AOw
IDw
DOw
OCw
Aww
Dww
Cww
BCw
ADw
ACw
ICw
DCw
ABw

Fig. 6

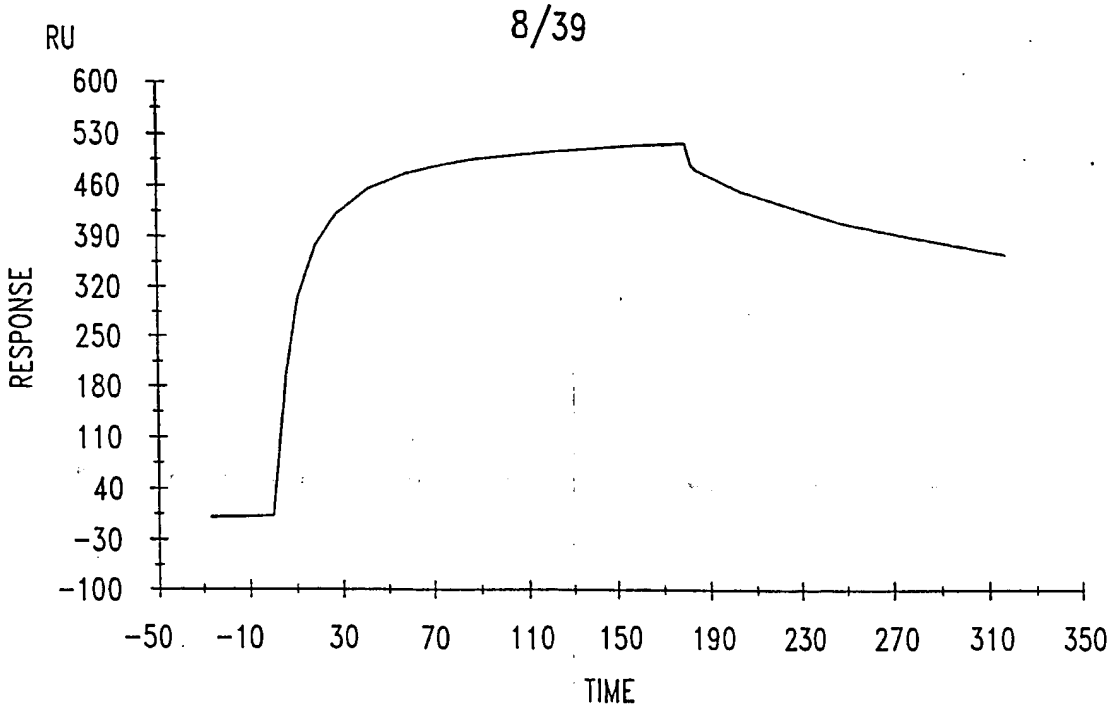

RU
600
530
460
390
320
250
180
110
40
-30
-100
RESPONSE
-50
-10
30
70
110
150
190
230
270
310
350
TIME

Fig. 7A

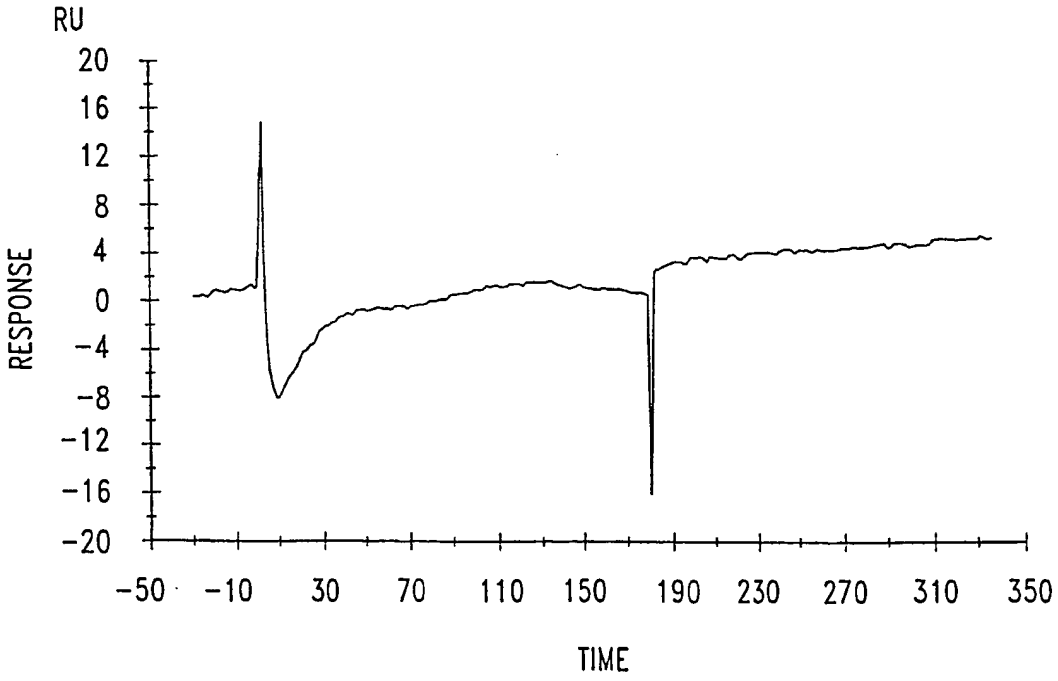

RU
20
16
12
8
4
0
-4
-8
-12
-16
-20
RESPONSE
-50
-10
30
70
110
150
190
230
270
310
350
TIME

Fig. 7B

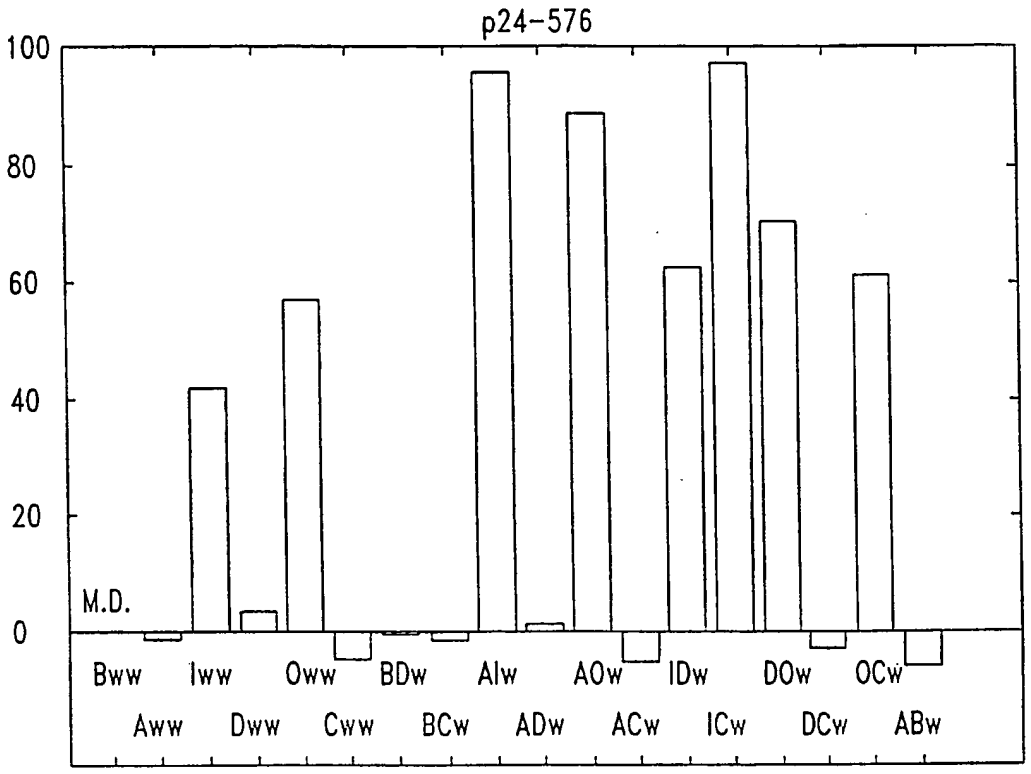

p24-576
100
80
60
40
20
0
M.D.
Bww
Iww
Oww
BDw
Alw
AOw
IDw
DOw
OCw
Aww
Dww
Cww
BCw
ADw
ACw
ICw
DCw
ABw

Fig. 8

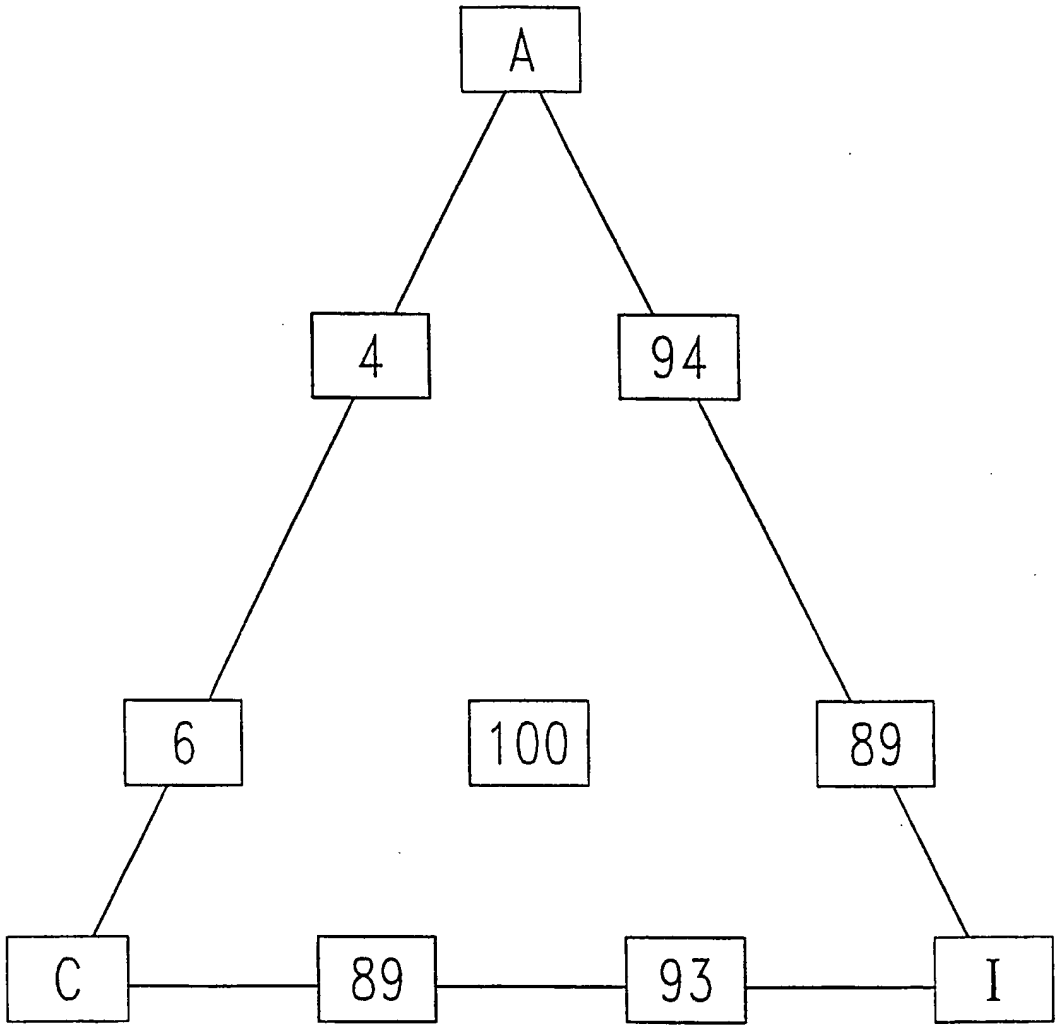


Fig. 9

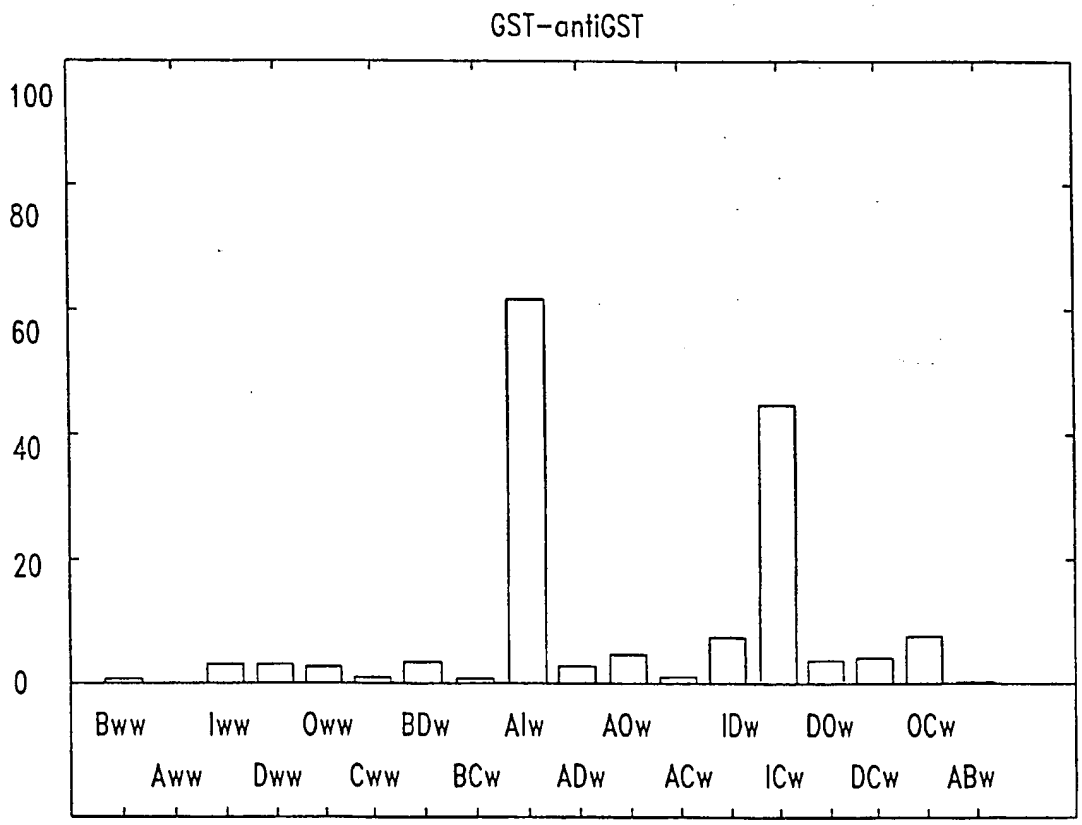

GST-antiGST
100
80
60
40
20
0
Bww
Iww
Oww
BDw
Alw
AOw
IDw
DOw
OCw
Aww
Dww
Cww
BCw
ADw
ACw
ICw
DCw
ABw

Fig. 10

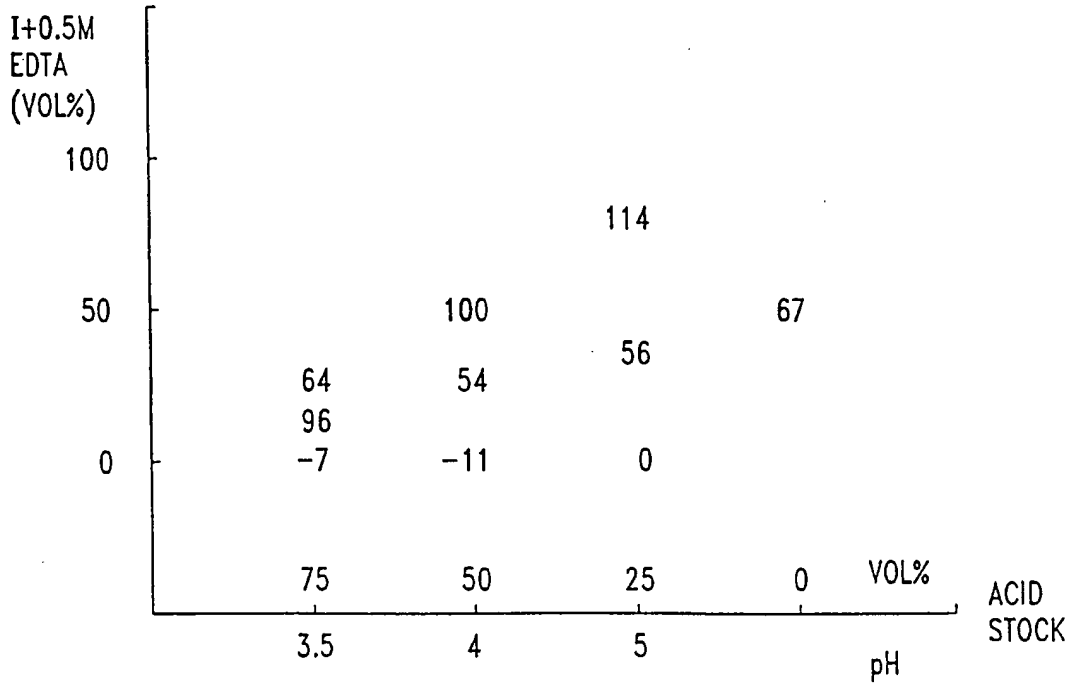

I+0.5M
EDTA
(VOL%)
100
50
0
114
100
67
56
64
54
96
−7
−11
0
75
50
25
0
VOL%
ACID
STOCK
3.5
4
5
pH

Fig. 11

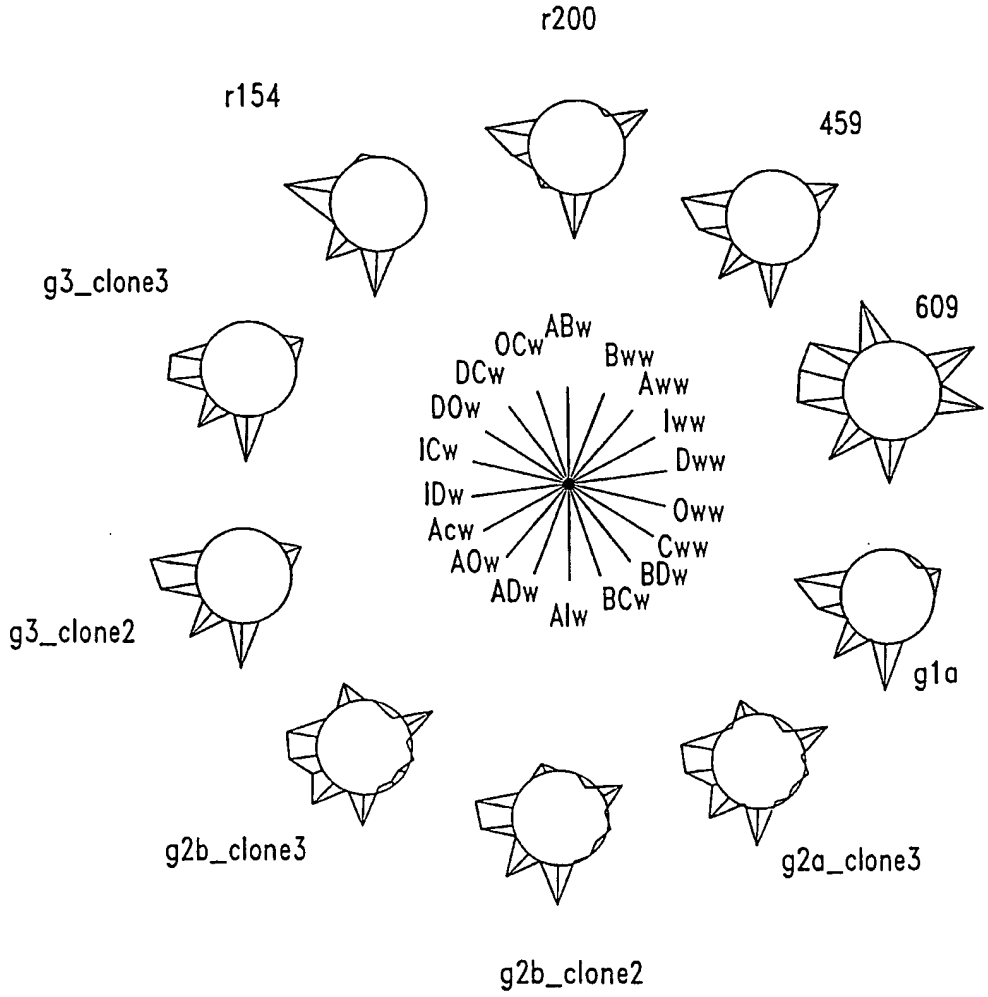

r200
r154
459
g3_clone3
609
OCw
ABw
Bww
DCw
Aww
DOw
Iww
ICw
Dww
IDw
Oww
Acw
Cww
AOw
BDw
ADw
BCw
Alw
g3_clone2
g1a
g2b_clone3
g2a_clone3
g2b_clone2

Fig. 12

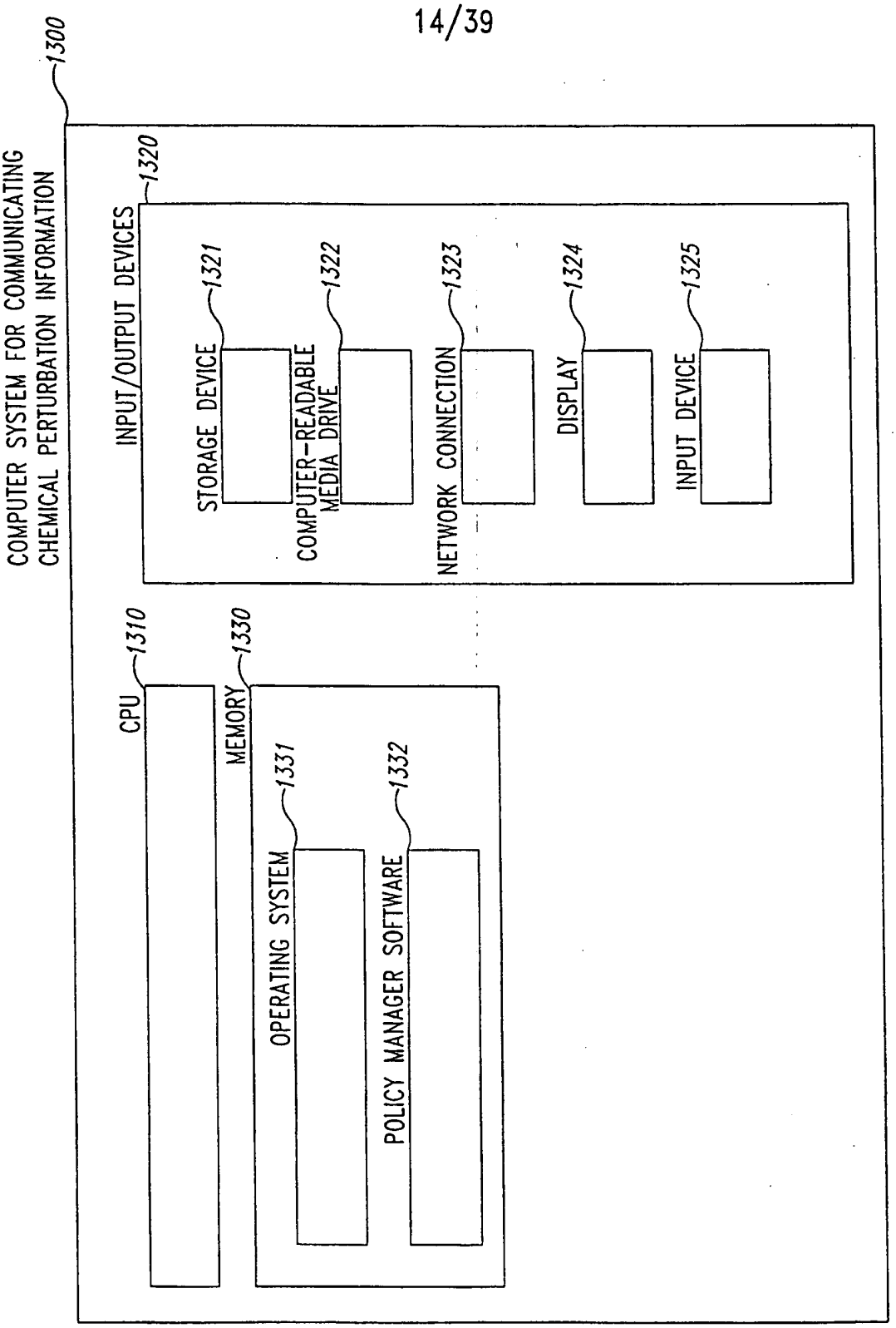
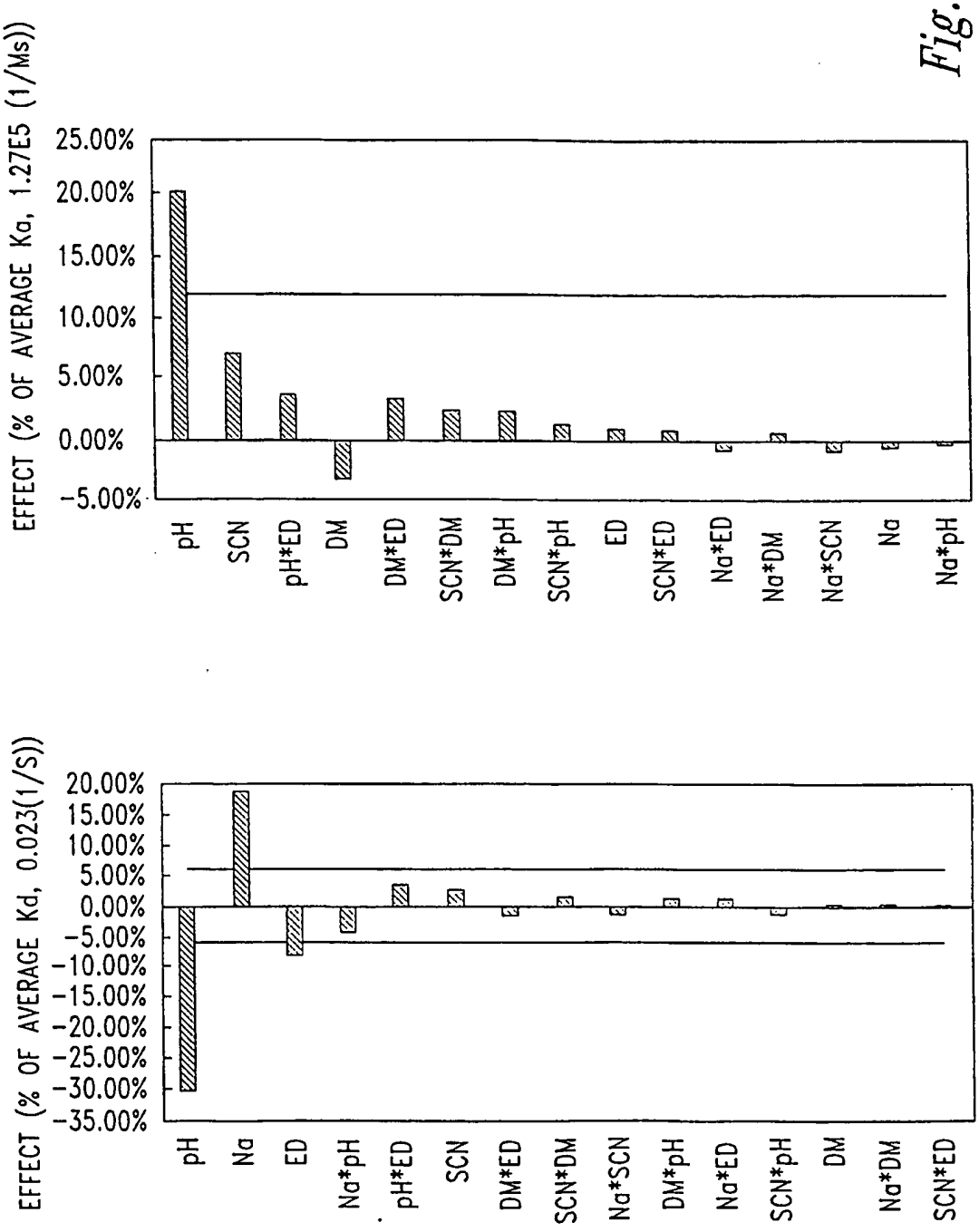

1300
COMPUTER SYSTEM FOR COMMUNICATING CHEMICAL PERTURBATION INFORMATION
1320
INPUT/OUTPUT DEVICES
1321
STORAGE DEVICE
1322
COMPUTER-READABLE MEDIA DRIVE
1323
NETWORK CONNECTION
1324
DISPLAY
1325
INPUT DEVICE
1310
CPU
1330
MEMORY
1331
OPERATING SYSTEM
1332
POLICY MANAGER SOFTWARE

Fig. 13

Fig. 14


EFFECT (% OF AVERAGE Ka, 1.27E5 (1/Ms))
25.00%
20.00%
15.00%
10.00%
5.00%
0.00%
-5.00%
pH
SCN
pH*ED
DM
DM*ED
SCN*DM
DM*pH
SCN*pH
ED
SCN*ED
Na*ED
Na*DM
Na*SCN
Na
Na*pH
EFFECT (% OF AVERAGE Kd, 0.023(1/S))
20.00%
15.00%
10.00%
5.00%
0.00%
-5.00%
-10.00%
-15.00%
-20.00%
-25.00%
-30.00%
-35.00%
pH
Na
ED
Na*pH
pH*ED
SCN
DM*ED
SCN*DM
Na*SCN
DM*pH
Na*ED
SCN*pH
DM
Na*DM
SCN*ED

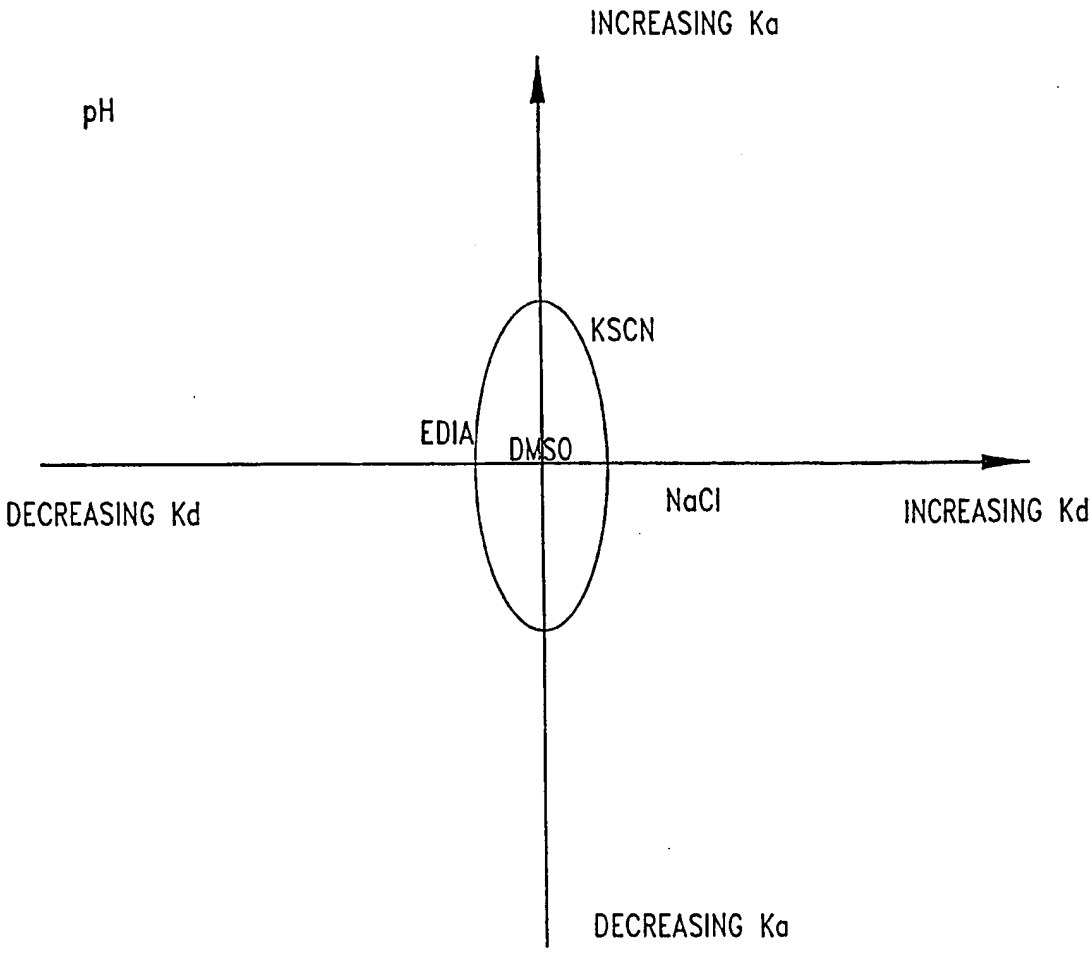

INCREASING Ka
pH
KSCN
EDIA
DMSO
NaCl
DECREASING Kd
INCREASING Kd
DECREASING Ka

Fig. 15

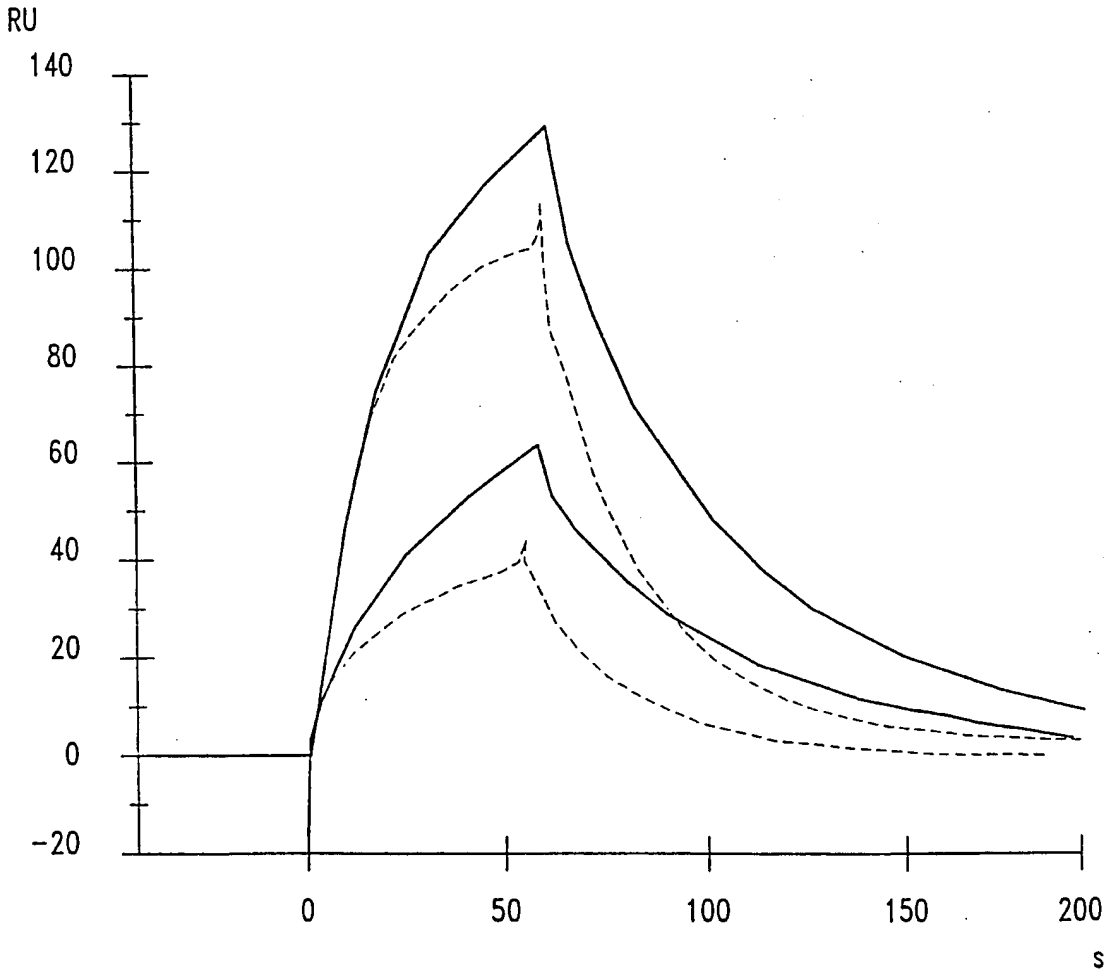
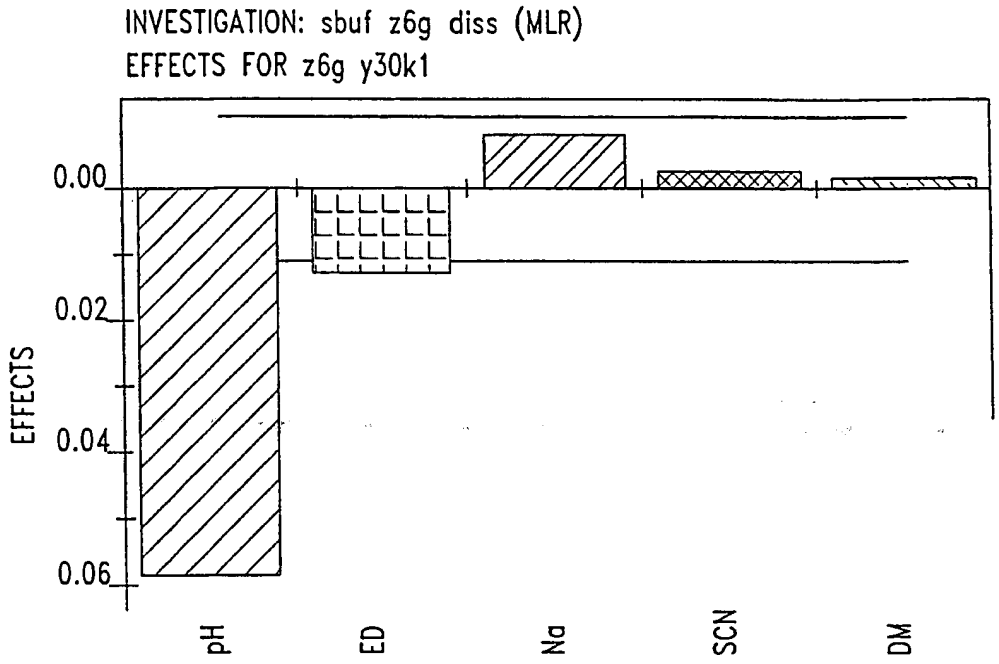
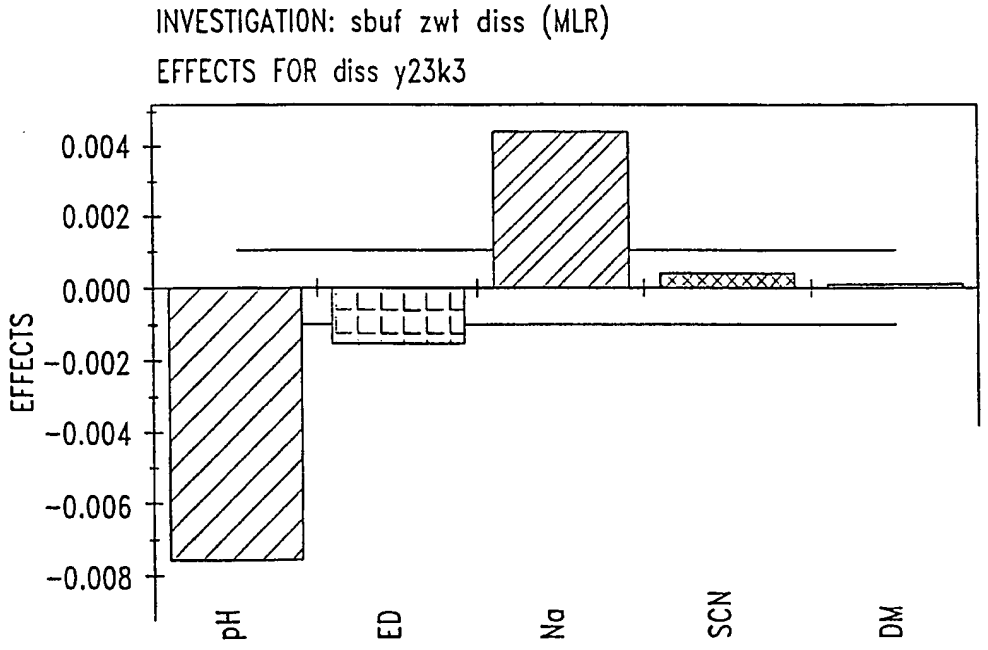


Fig. 16



N=17 R2=0.9385 R2Adj=0.9106
DF=11 Q2=0.8340 RSD=0.0092 ConfLev=0.95

Fig. 17



N=16 R2=0.9744 R2Adj=0.9616
DF=10 Q2=0.9345 RSD=0.0092 ConfLev=0.95

Fig. 18

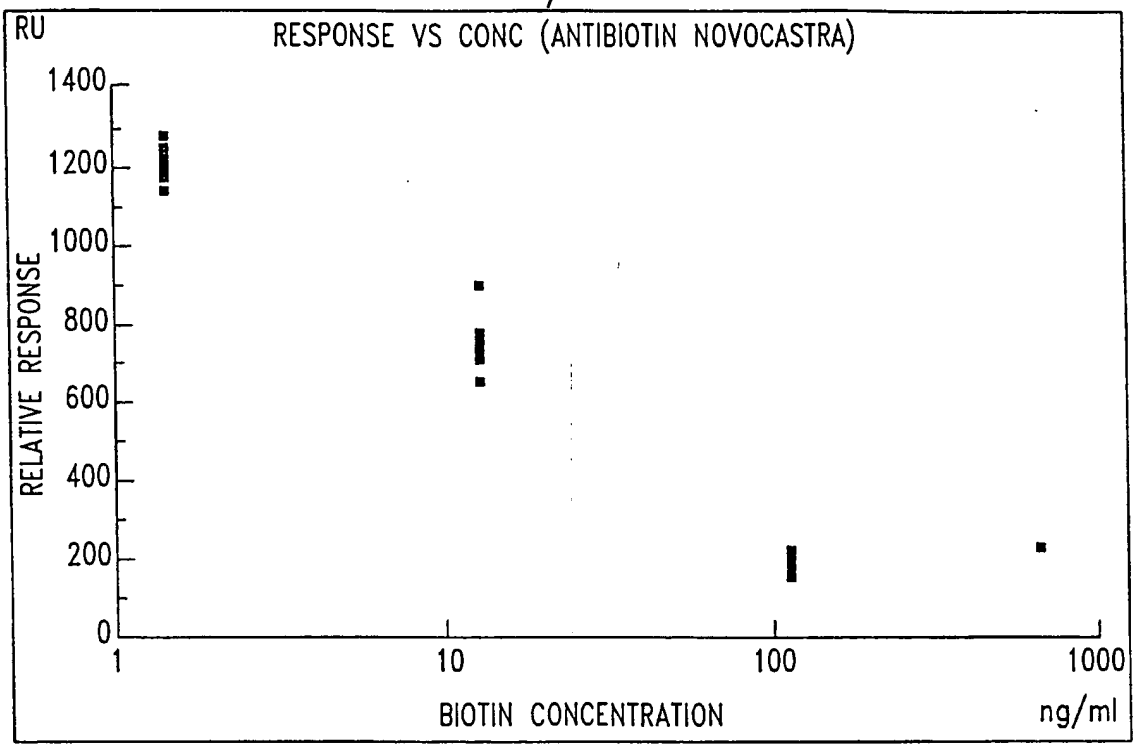


Fig. 19A

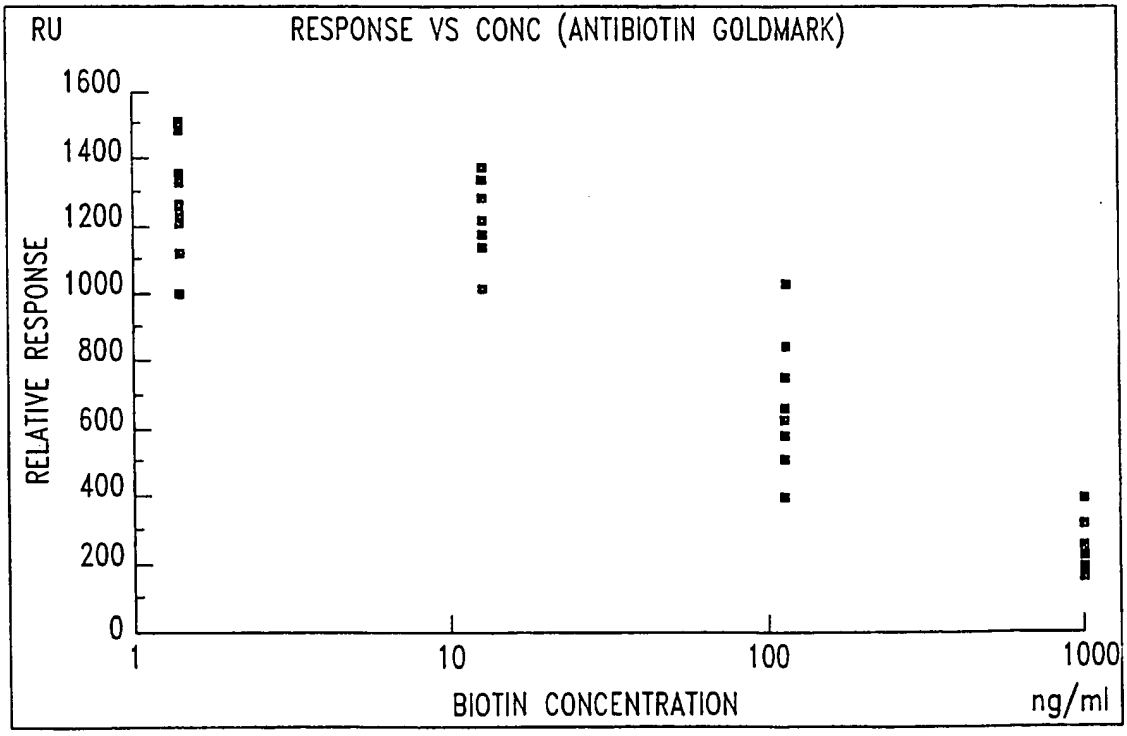


Fig. 19B

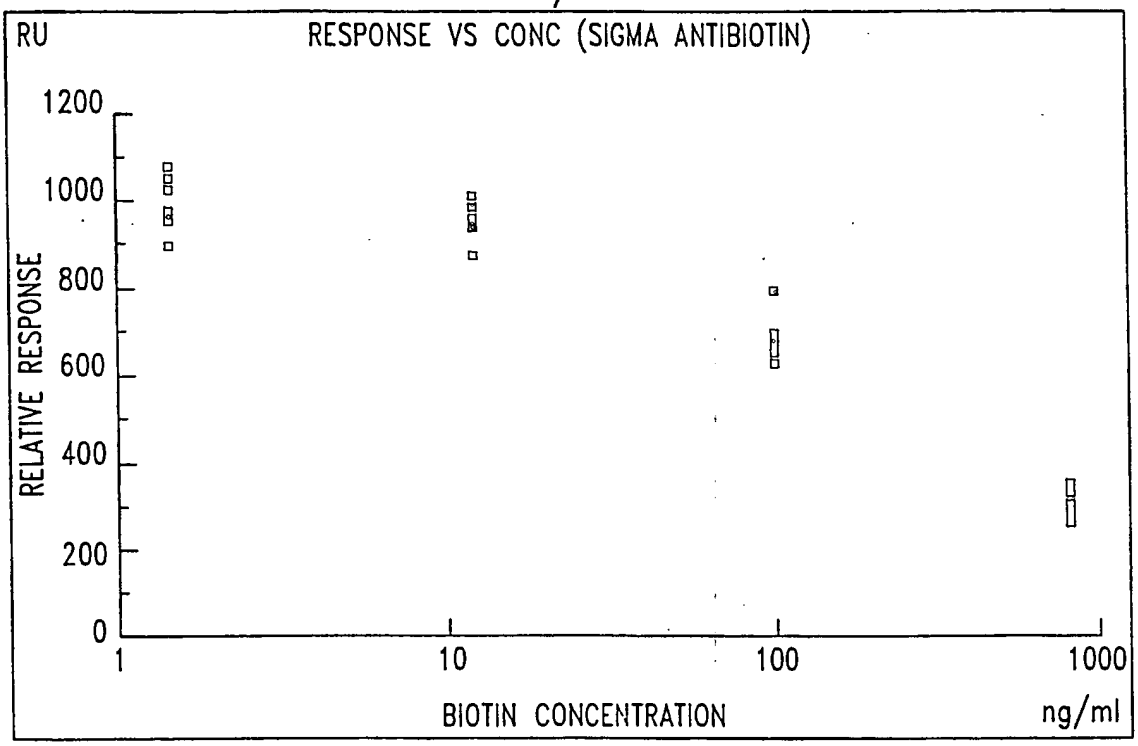


Fig. 19C

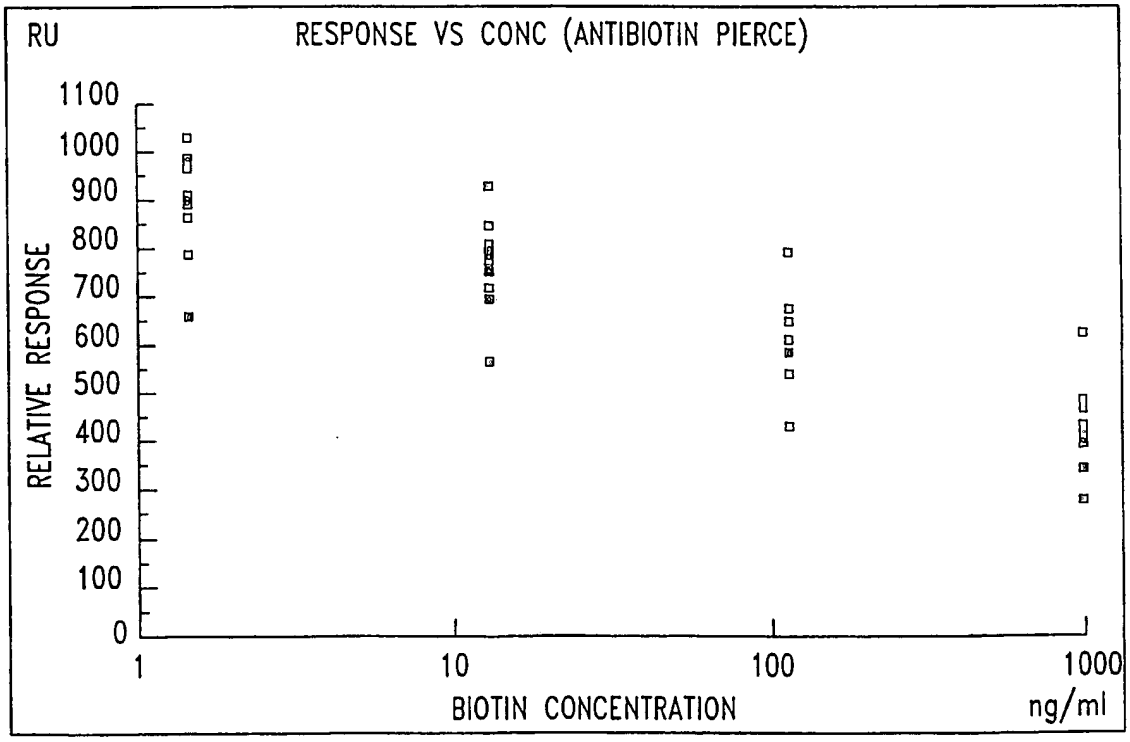


Fig. 19D

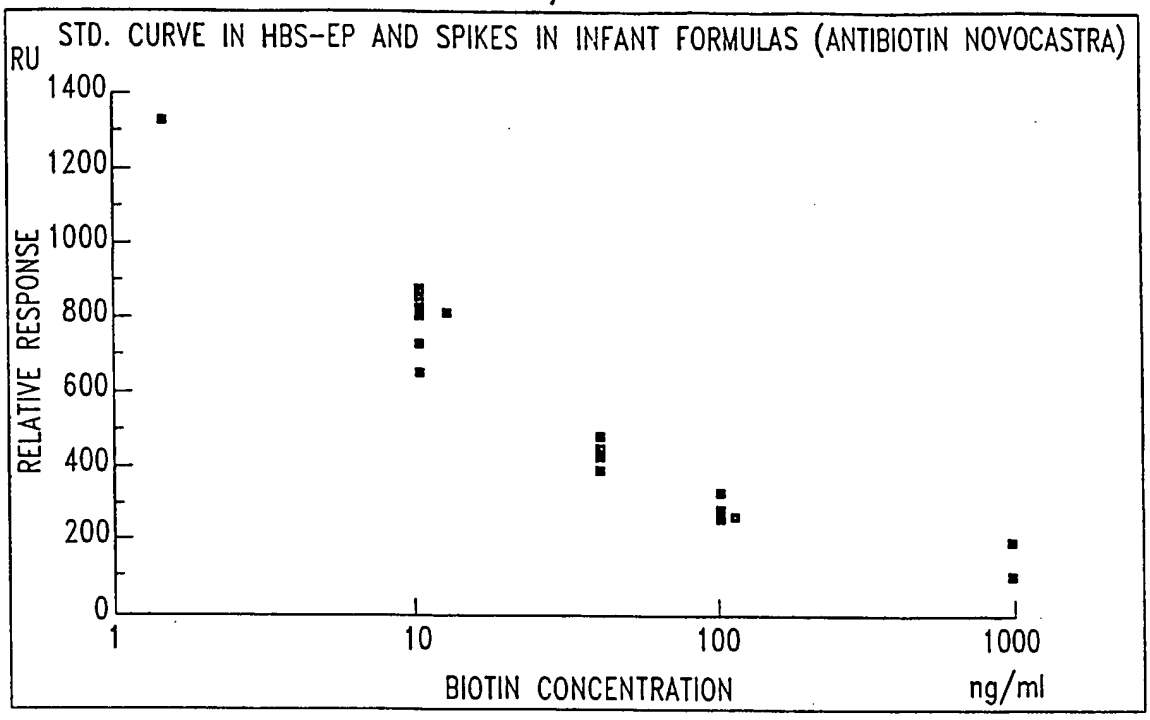


Fig. 20A

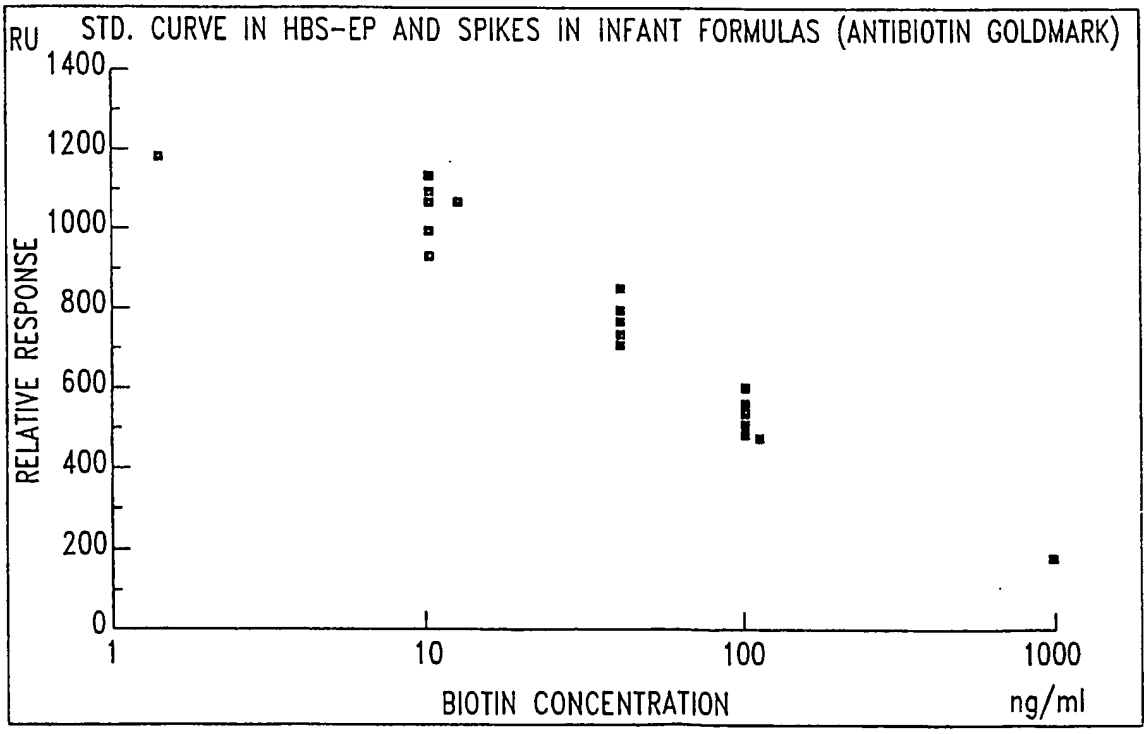


Fig. 20B

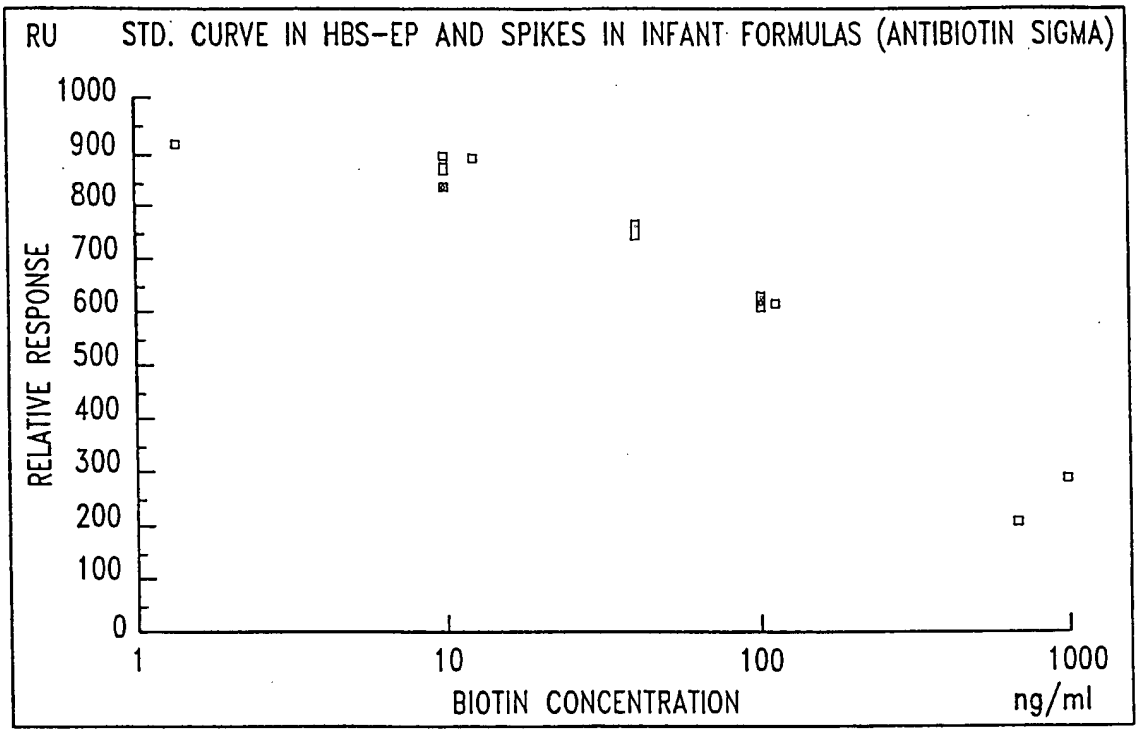


Fig. 20C

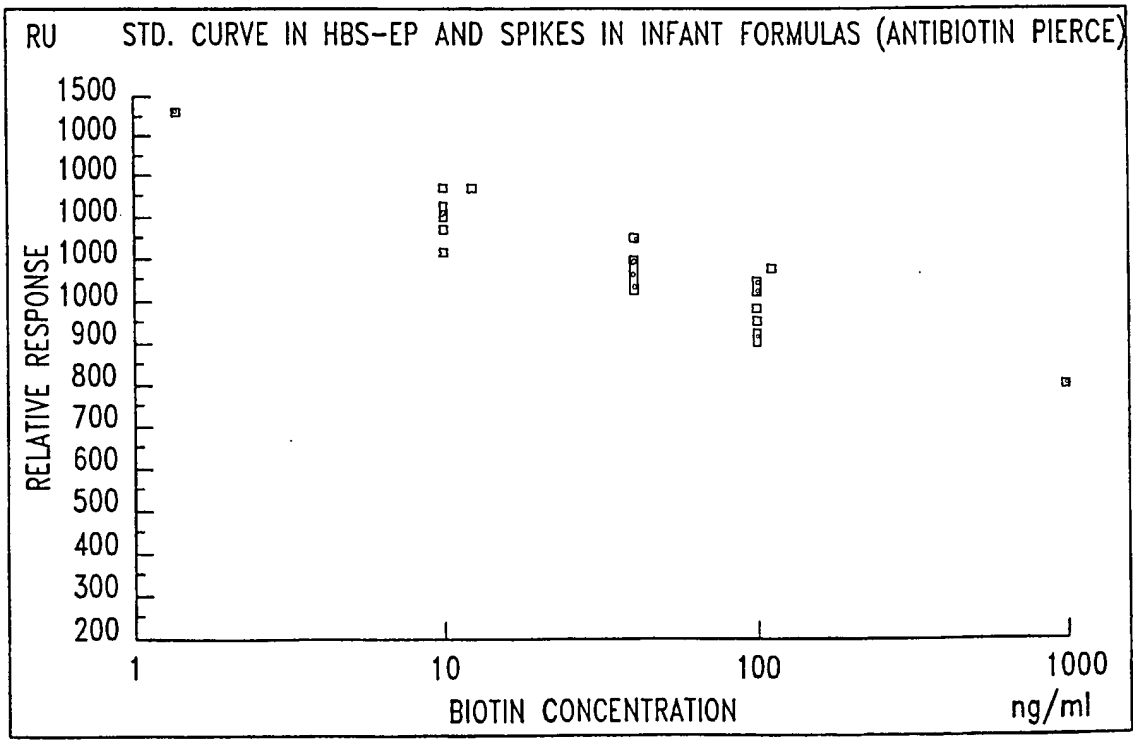


Fig. 20D

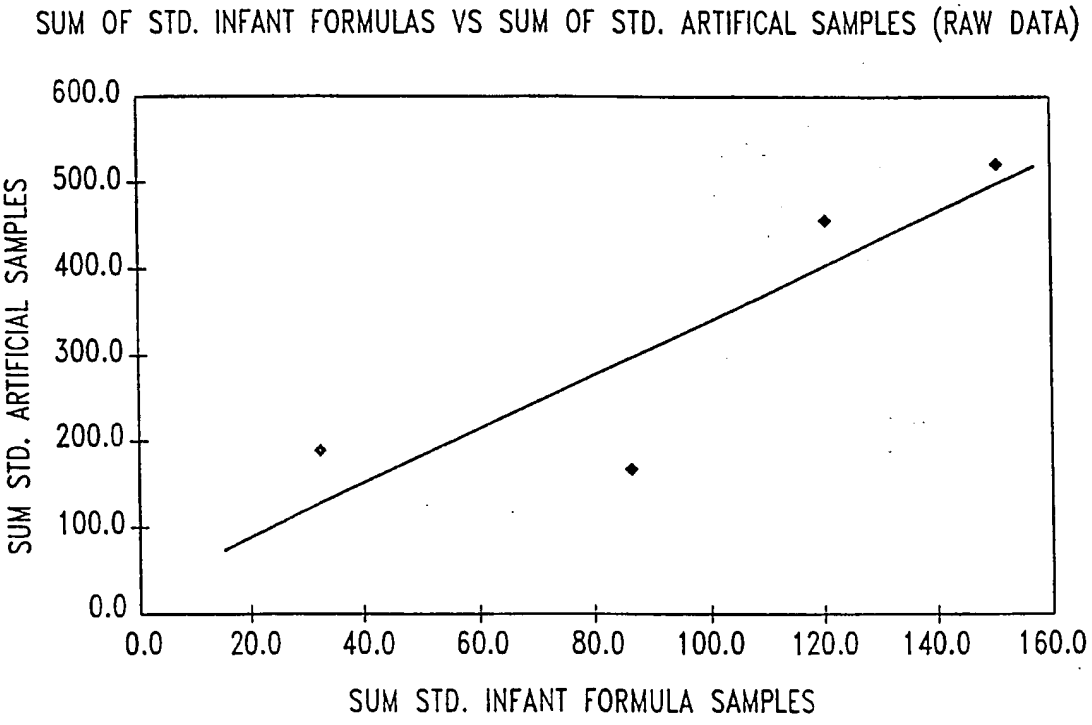

SUM OF STD. INFANT FORMULAS VS SUM OF STD. ARTIFICAL SAMPLES (RAW DATA)
SUM STD. ARTIFICIAL SAMPLES
600.0
500.0
400.0
300.0
200.0
100.0
0.0
0.0
20.0
40.0
60.0
80.0
100.0
120.0
140.0
160.0
SUM STD. INFANT FORMULA SAMPLES

Fig. 21

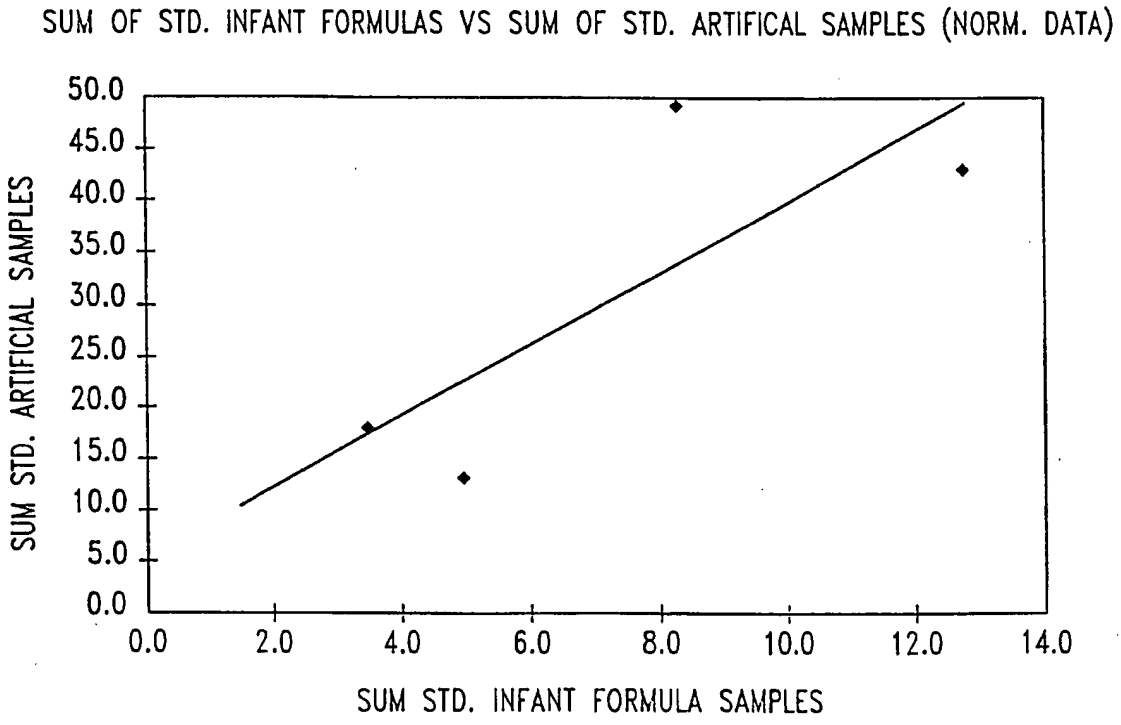

SUM OF STD. INFANT FORMULAS VS SUM OF STD. ARTIFICAL SAMPLES (NORM. DATA)
SUM STD. ARTIFICIAL SAMPLES
50.0
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
0.0
2.0
4.0
6.0
8.0
10.0
12.0
14.0
SUM STD. INFANT FORMULA SAMPLES

Fig. 22

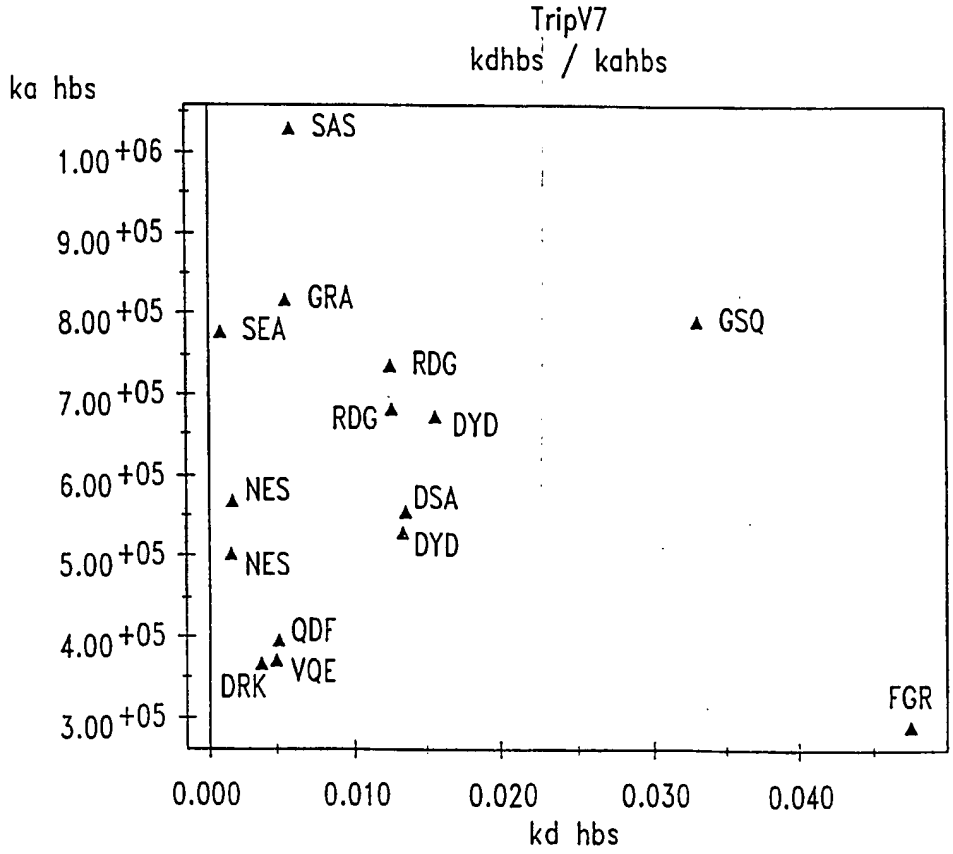

TripV7
kdhbs / kahbs
ka hbs
1.00+06
9.00+05
8.00+05
7.00+05
6.00+05
5.00+05
4.00+05
3.00+05
SAS
GRA
SEA
RDG
RDG
DYD
GSQ
NES
DSA
DYD
NES
QDF
VQE
DRK
FGR
0.000
0.010
0.020
0.030
0.040
kd hbs
Simca-P 7.01 by Umetri
AB 1999-04-26 10:36

Fig. 23

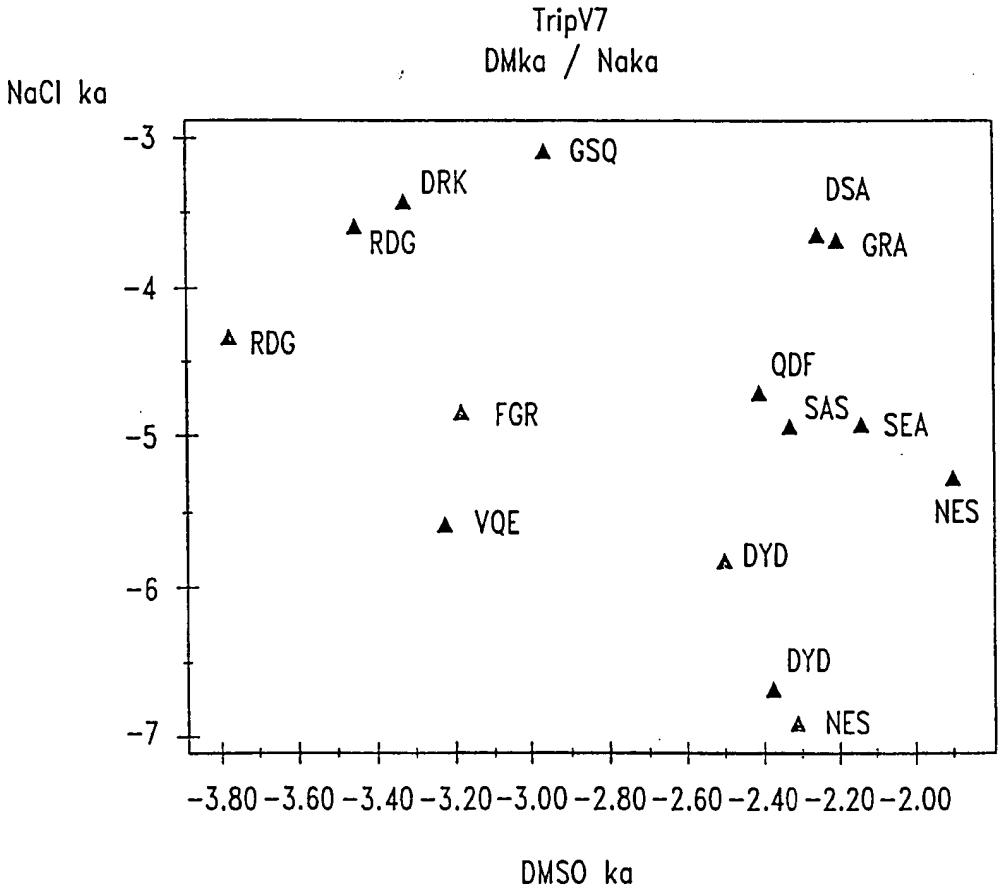

TripV7
DMka / Naka
NaCl ka
-3
-4
-5
-6
-7
GSQ
DRK
RDG
DSA
GRA
RDG
QDF
FGR
SAS
SEA
NES
VQE
DYD
DYD
NES
-3.80 -3.60 -3.40 -3.20 -3.00 -2.80 -2.60 -2.40 -2.20 -2.00
DMSO ka
Simca-P 7.01 by Umetri
AB 1999-04-25

Fig. 24

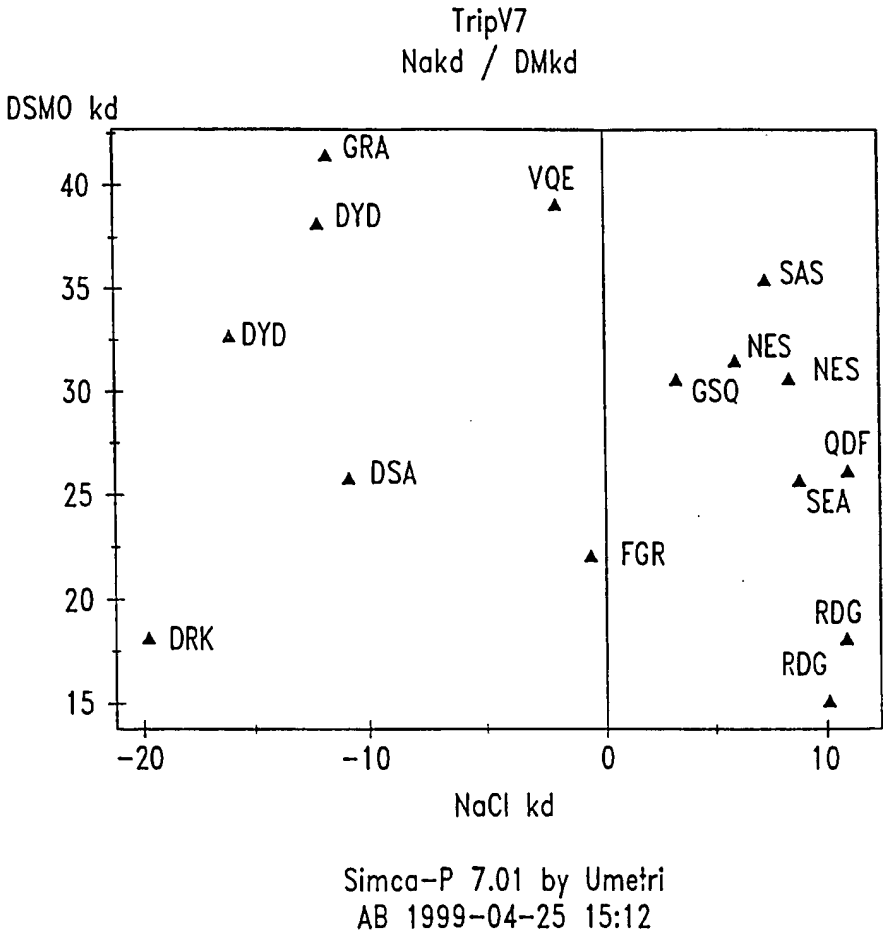

TripV7
Nakd / DMkd
DSMO kd
40
35
30
25
20
15
GRA
VQE
DYD
DYD
SAS
NES
NES
GSQ
QDF
DSA
SEA
FGR
RDG
DRK
RDG
−20
−10
0
10
NaCl kd
Simca−P 7.01 by Umetri
AB 1999−04−25 15:12

Fig. 25

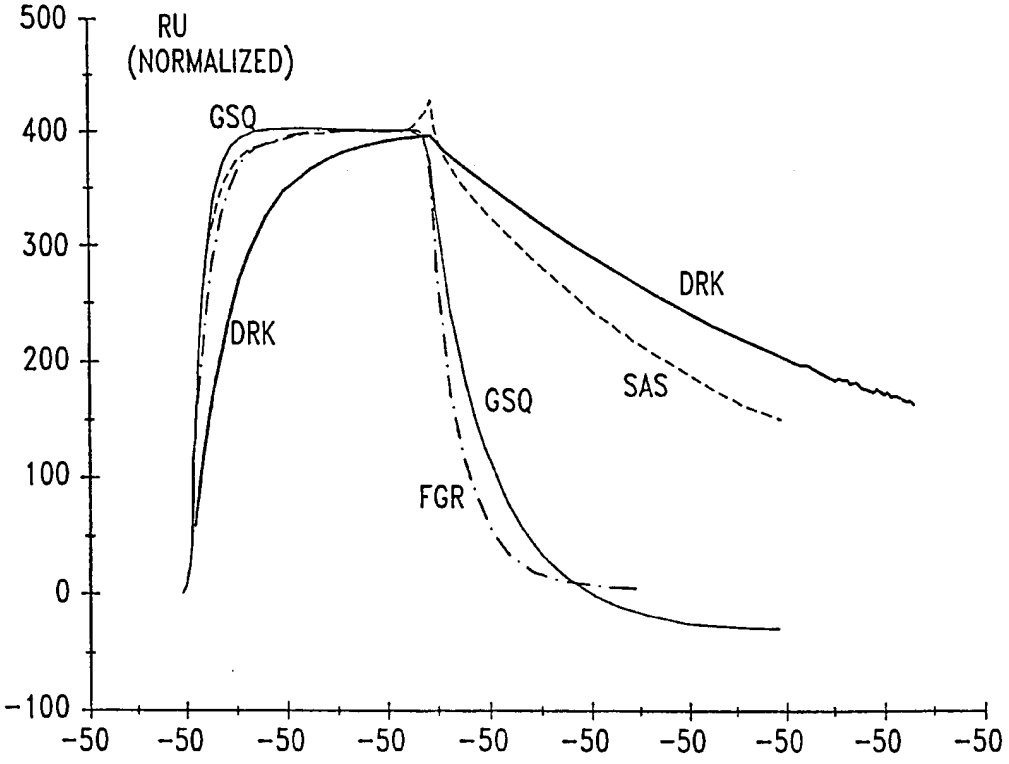

500
400
300
200
100
0
-100
RU
(NORMALIZED)
GSQ
DRK
DRK
SAS
GSQ
FGR
-50
-50
-50
-50
-50
-50
-50
-50
-50
-50

Fig. 26

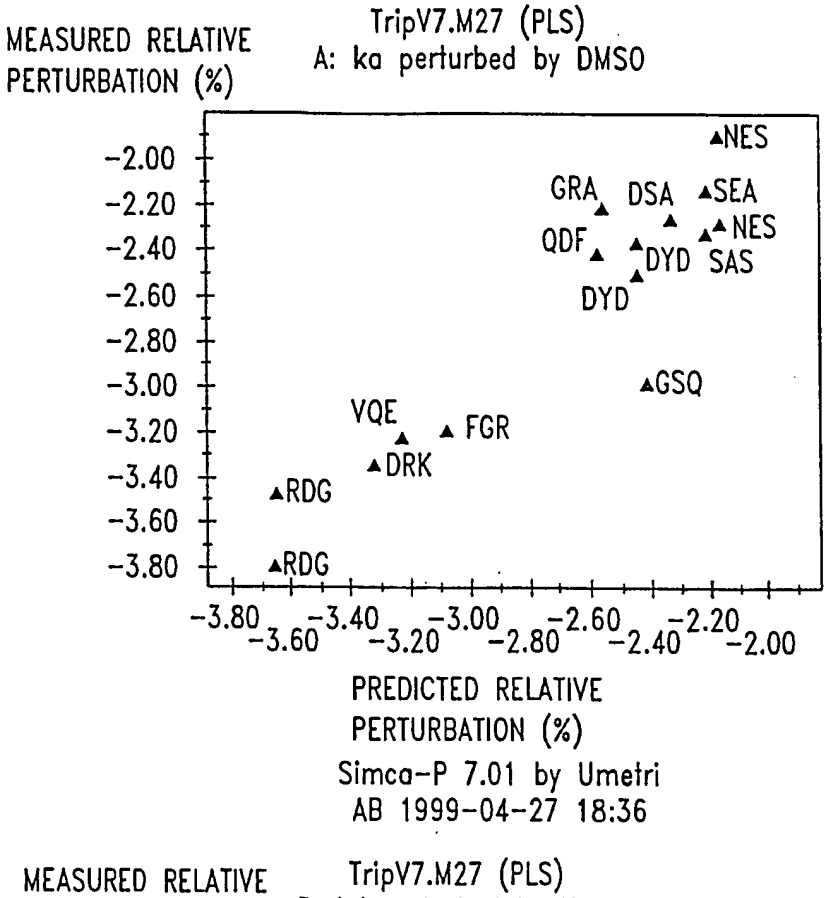


Fig. 27A

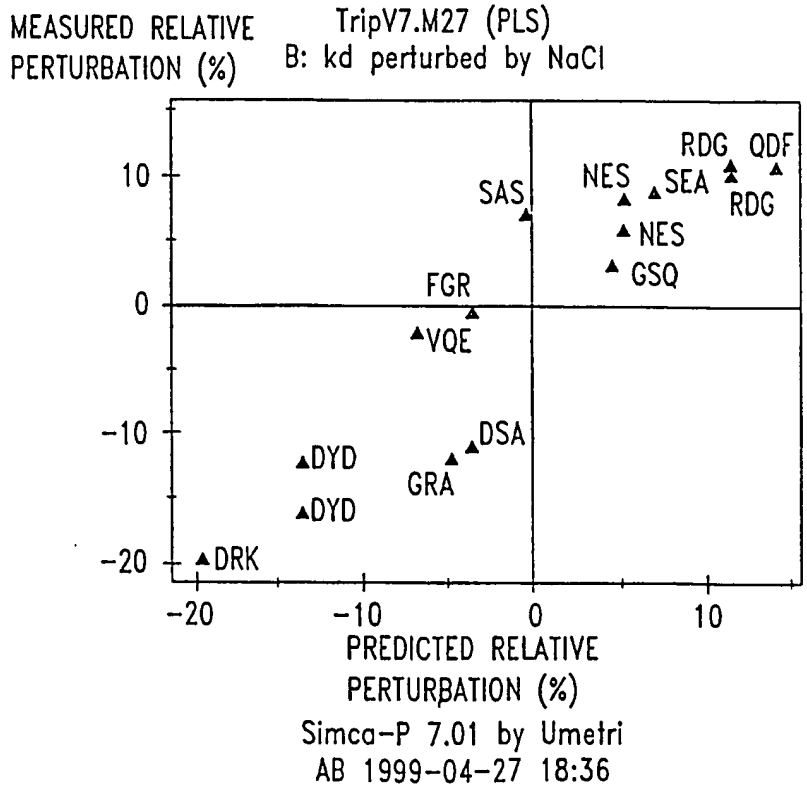


Fig. 27B

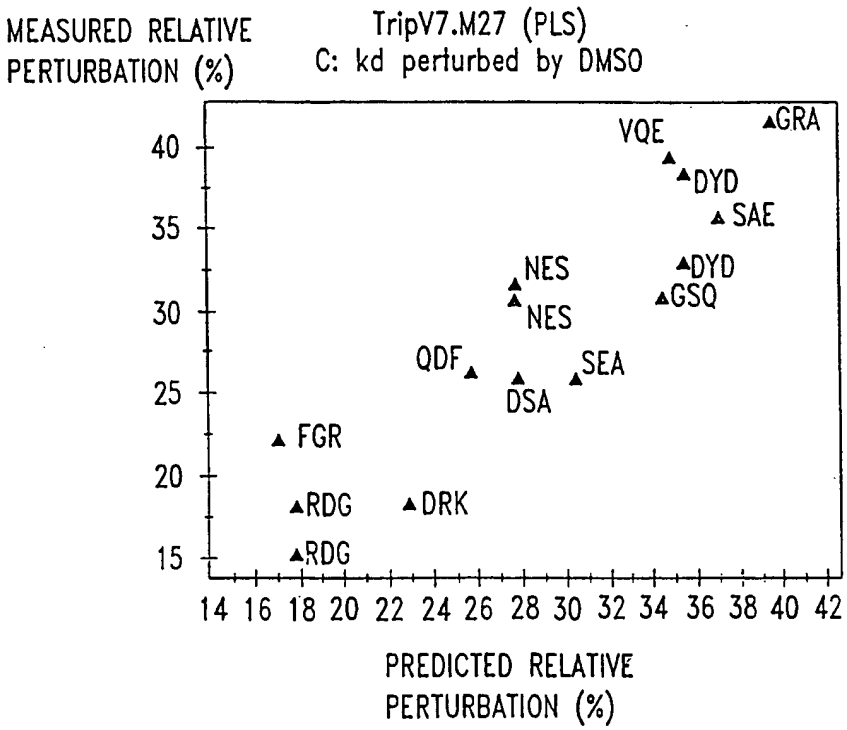

MEASURED RELATIVE PERTURBATION (%)
TripV7.M27 (PLS)
C: kd perturbed by DMSO
GRA
VQE
DYD
SAE
NES
DYD
GSQ
NES
QDF
SEA
DSA
FGR
RDG
DRK
RDG
40
35
30
25
20
15
14 16 18 20 22 24 26 28 30 32 34 36 38 40 42
PREDICTED RELATIVE PERTURBATION (%)
Simca-P 7.01 by Umetri AB 1999-04-27 18:36

Fig. 27C

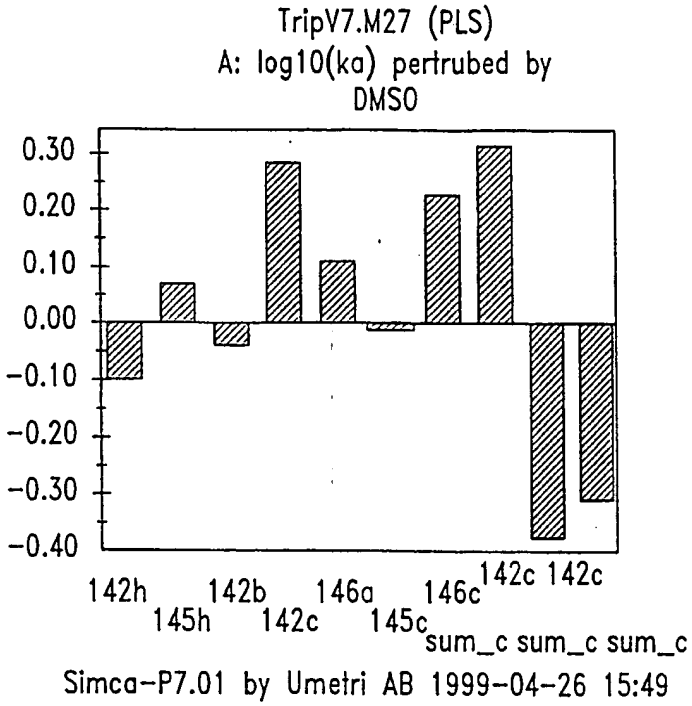


Fig. 28A

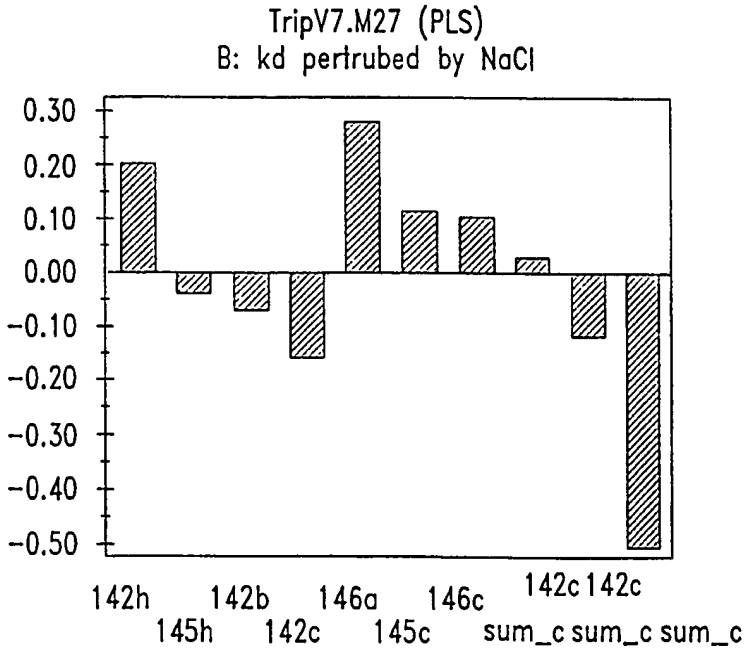


Fig. 28B

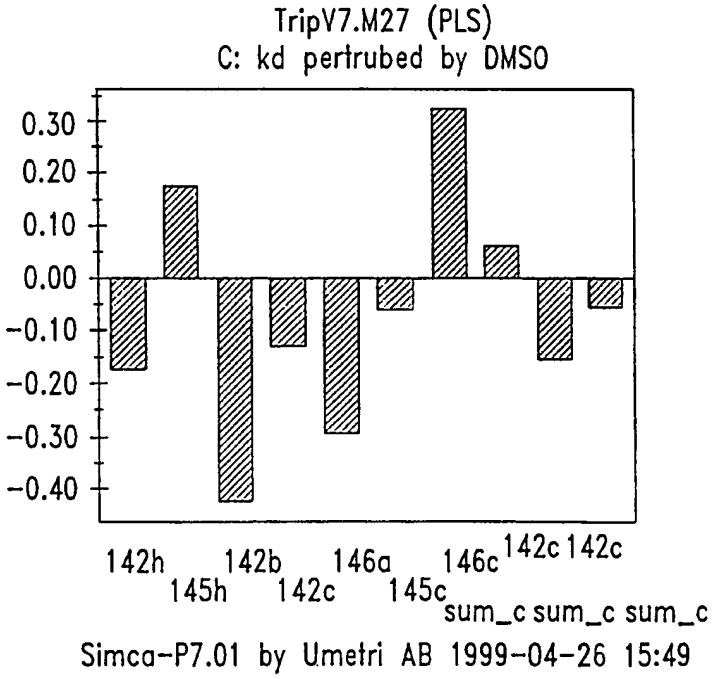
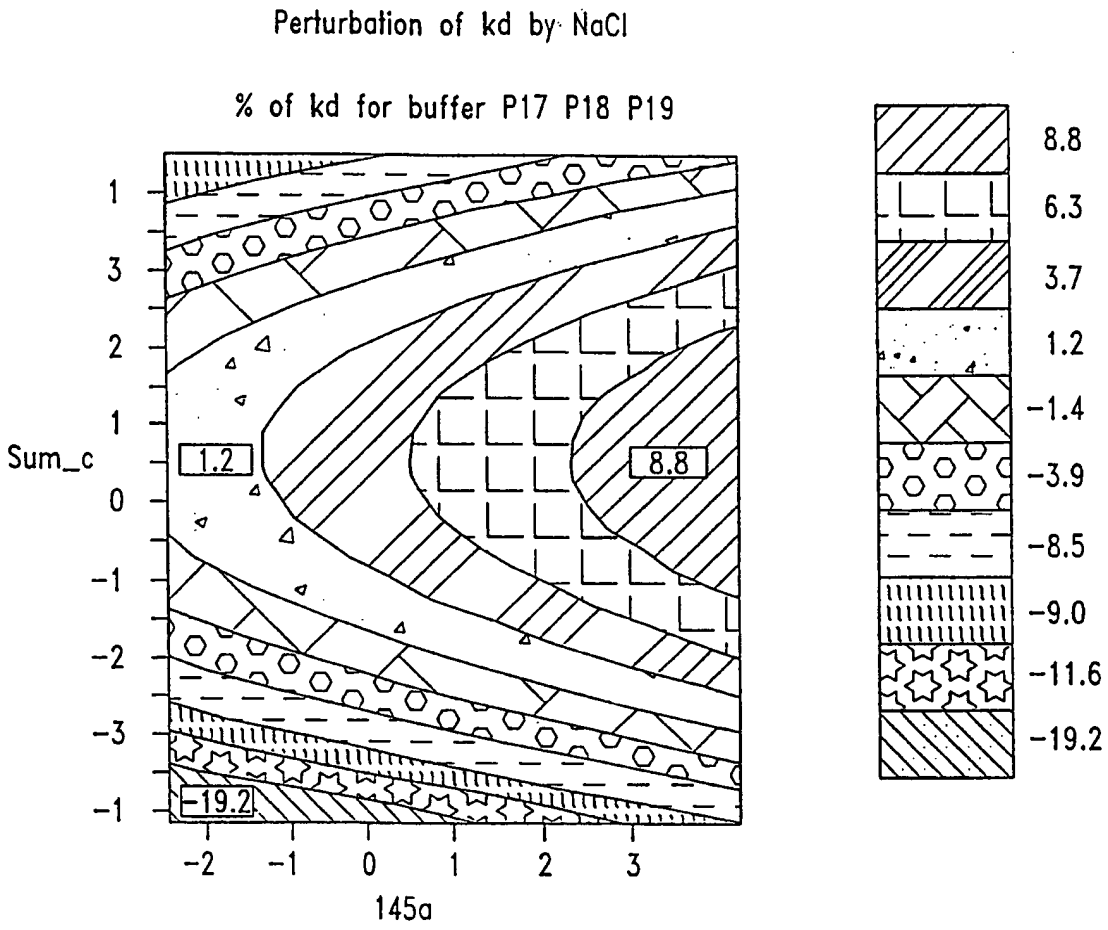

TripV7.M27 (PLS)
C: kd pertrubed by DMSO
0.30
0.20
0.10
0.00
-0.10
-0.20
-0.30
-0.40
142h
145h
142b
142c
146a
145c
146c
142c
sum_c
142c
sum_c
sum_c
Simca-P7.01 by Umetri AB 1999-04-26 15:49

Fig. 28C



SETTINGS OF THE OTHER DESCRIPTORS:	
DESCRIPTOR	VALUE
142h	0.070
145h	0.220
142b	0.100
142c	0.260
145c	0.076
146c	0.223

Fig. 29

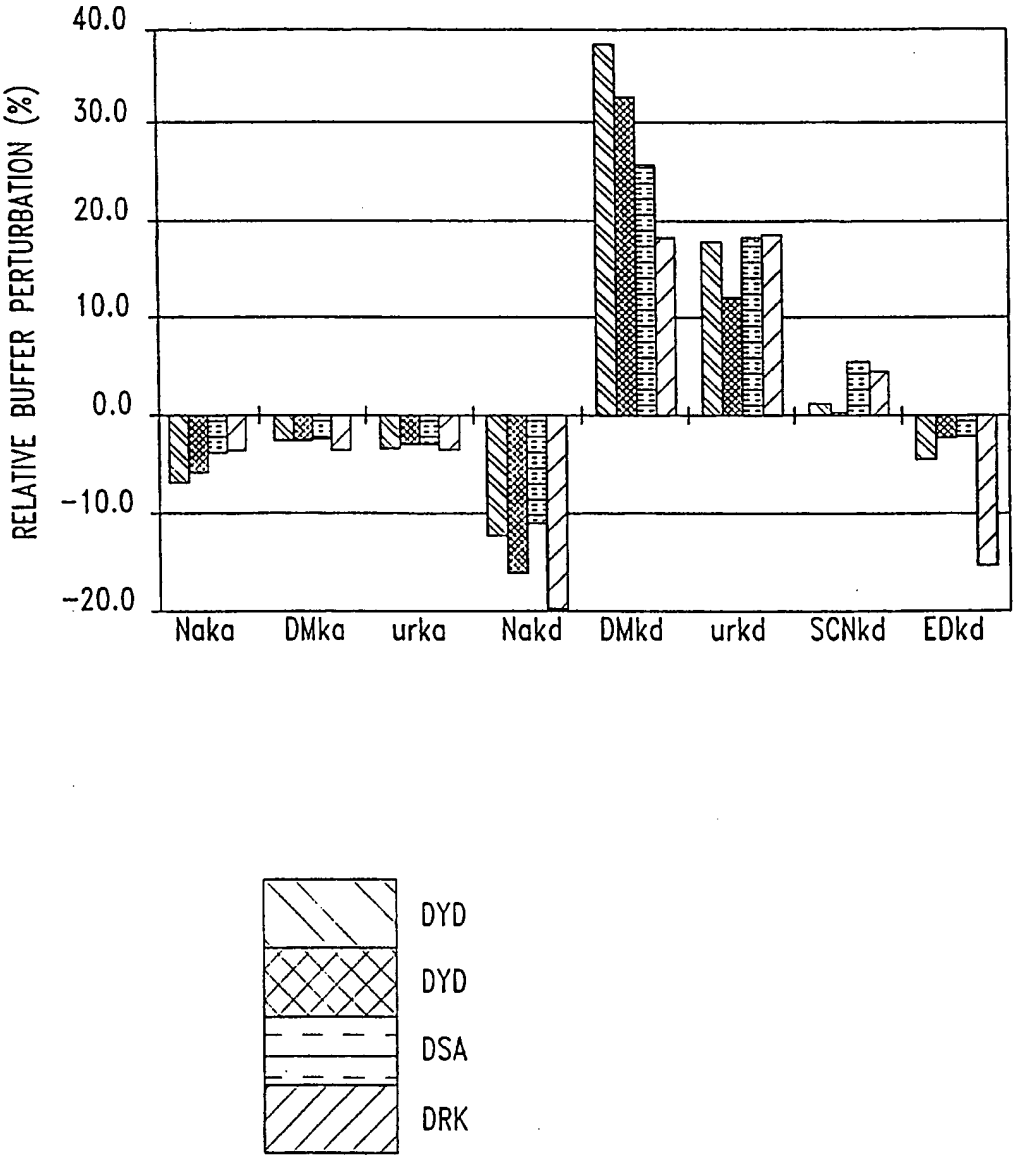

40.0
30.0
20.0
10.0
0.0
-10.0
-20.0
RELATIVE BUFFER PERTURBATION (%)
Naka
DMka
urka
Nakd
DMkd
urkd
SCNkd
EDkd
DYD
DYD
DSA
DRK

Fig. 30

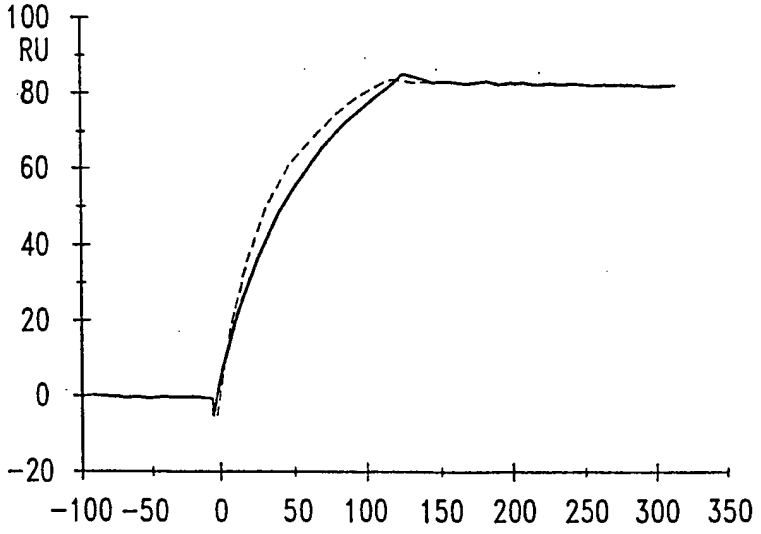


Fig. 31A

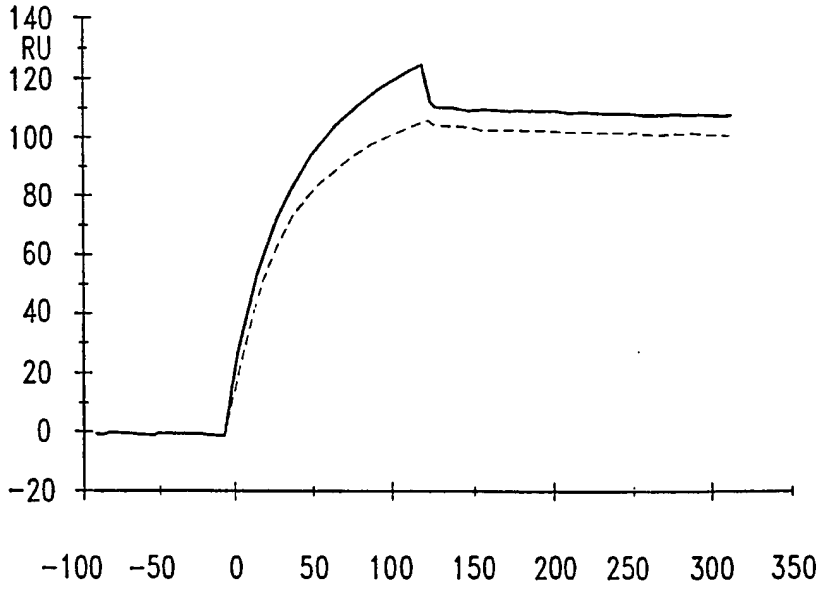


Fig. 31B

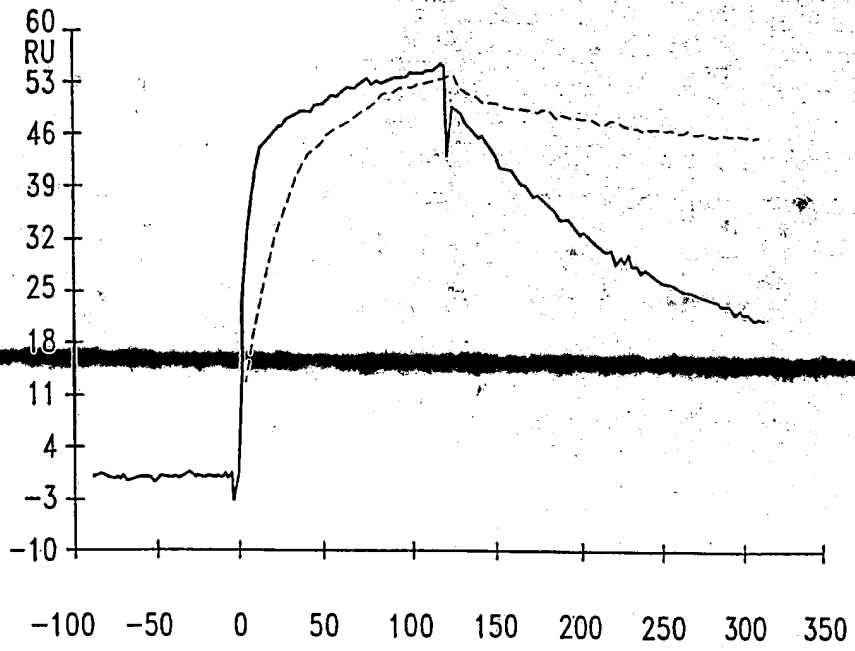


Fig. 32A

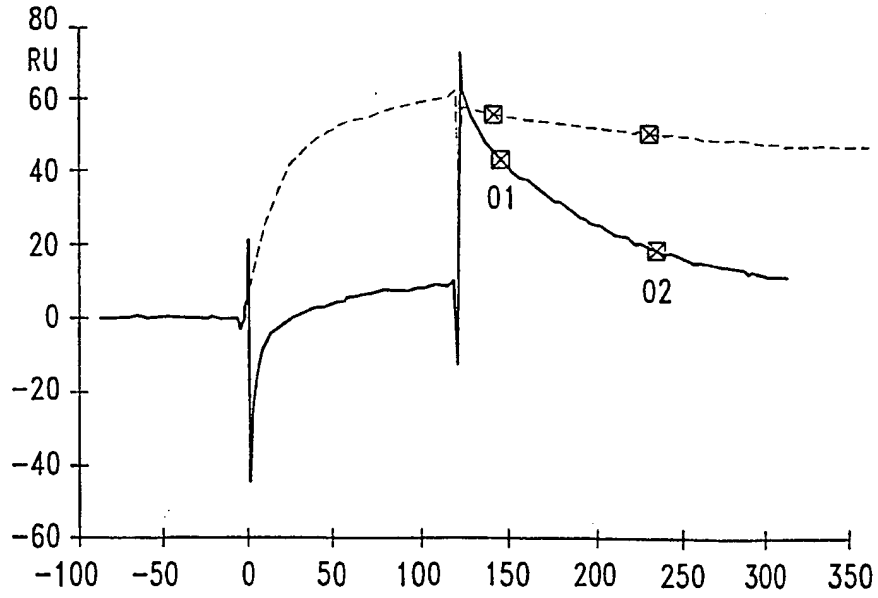


Fig. 32B

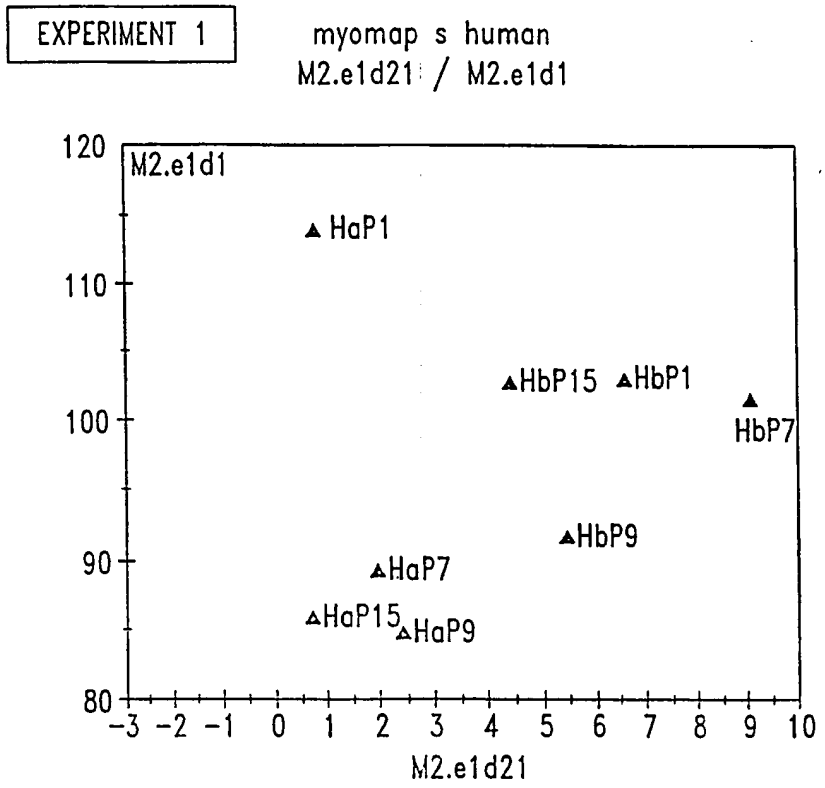

EXPERIMENT 1
myomap s human
M2.e1d21 / M2.e1d1
M2.e1d1
HaP1
HbP15
HbP1
HbP7
HbP9
HaP7
HaP15
HaP9
120
110
100
90
80
-3 -2 -1 0 1 2 3 4 5 6 7 8 9 10
M2.e1d21
Simca-P7.01 by Umetri AB 1999-05-07 14:56

Fig. 33A

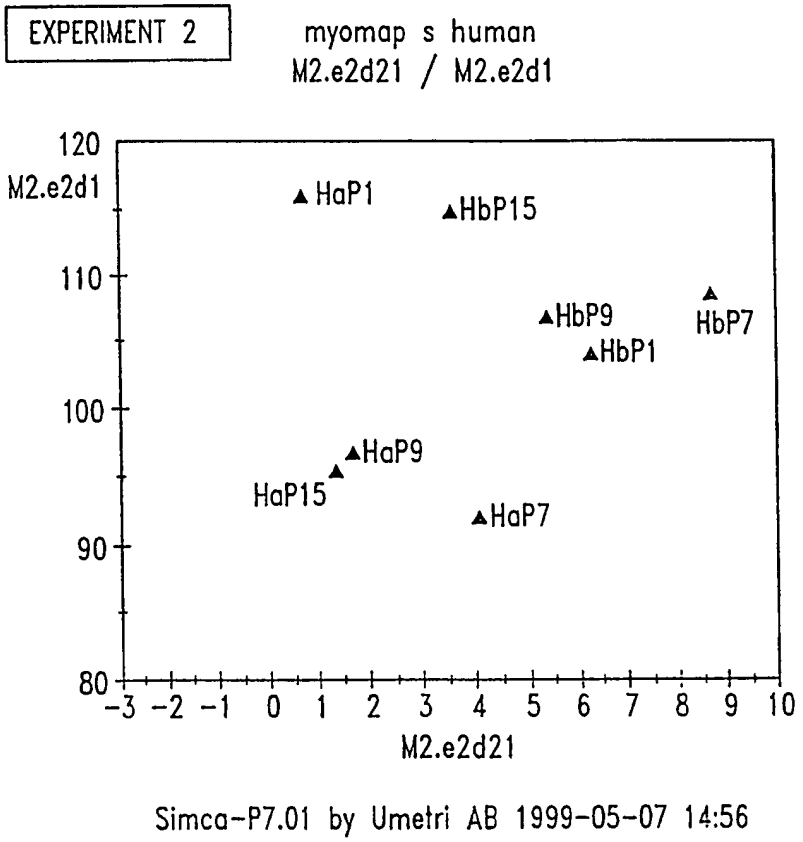

EXPERIMENT 2
myomap s human
M2.e2d21 / M2.e2d1
120
M2.e2d1
110
100
90
80
HaP1
HbP15
HbP9
HbP7
HbP1
HaP9
HaP15
HaP7
-3 -2 -1 0 1 2 3 4 5 6 7 8 9 10
M2.e2d21
Simca-P7.01 by Umetri AB 1999-05-07 14:56

Fig. 33B

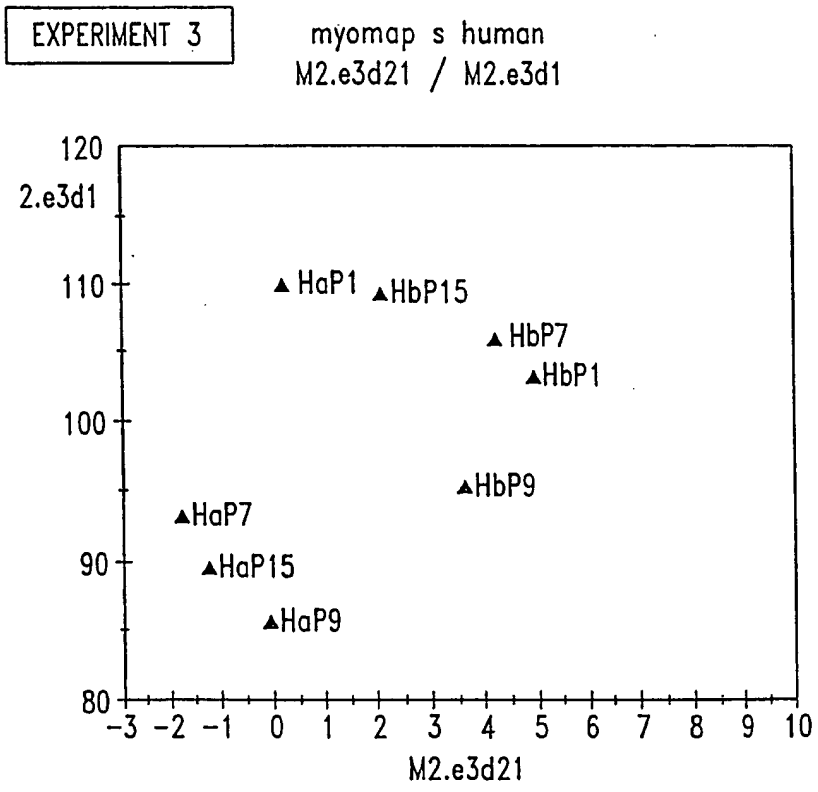

EXPERIMENT 3
myomap s human
M2.e3d21 / M2.e3d1
120
2.e3d1
110
100
90
80
HaP1
HbP15
HbP7
HbP1
HbP9
HaP7
HaP15
HaP9
-3 -2 -1 0 1 2 3 4 5 6 7 8 9 10
M2.e3d21
Simca-P7.01 by Umetri AB 1999-05-07 14:56

Fig. 33C

SEQUENCE LISTING

<110> Biacore

<120> SURFACE REGENERATION OF BIOSENSORS AND
CHARACTERIZATION OF BIOMOLECULES ASSOCIEATED THEREWITH

<130> 740073.402PC

<140> PCT
<141> 1999-05-28

<160> 15

<170> FastSEQ for Windows Version 3.0

<210> 1
<211> 58
<212> PRT
<213> Staphylococcus sp.

<400> 1

```
Val Asp Asn Lys Phe Asn Lys Glu Gln Gln Asn Ala Phe Tyr Glu Ile
 1               5                  10                  15
Leu His Leu Pro Asn Leu Asn Glu Glu Gln Arg Asn Ala Phe Ile Gln
            20                  25                  30
Ser Leu Lys Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
        35                  40                  45
Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
    50                  55
```

<210> 2
<211> 64
<212> PRT
<213> Artificial Sequence

<220>
<223> Mutated analogue of Staphylococcal Z fragment of protein A

<400> 2

```
Val Asp Asn Lys Phe Asn Lys Glu Gln Gln Asn Ala Phe Tyr Glu Ile
 1               5                  10                  15
Leu His Leu Pro Asn Leu Asn Glu Glu Gln Arg Asn Ala Phe Ile Gln
            20                  25                  30
Ser Leu Lys Asp Asp Pro Ser Gln Gly Gly Gly Gly Gly Gly Ser Ala
        35                  40                  45
Asn Leu Leu Ala Glu Ala Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
    50                  55                  60
```

<210> 3
<211> 16
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Peptide based on the wild type tobacco mosaic virus protein

<400> 3
Cys Gly Ser Tyr Asn Arg Ser Ser Phe Glu Ser Ser Ser Gly Leu Val
1 5 10 15

<210> 4
<211> 16
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Peptide based on the wild type tobacco mosaic virus protein

<400> 4
Cys Gly Ser Tyr Asn Arg Val Ser Phe Gln Glu Ser Ser Gly Leu Val
1 5 10 15

<210> 5
<211> 16
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Peptide based on the wild type tobacco mosaic virus protein

<400> 5
Cys Gly Ser Tyr Asn Arg Asp Ser Phe Tyr Asp Ser Ser Gly Leu Val
1 5 10 15

<210> 6
<211> 16
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Peptide based on the wild type tobacco mosaic virus protein

<400> 6
Cys Gly Ser Tyr Asn Arg Gly Ser Phe Arg Ala Ser Ser Gly Leu Val
1 5 10 15

<210> 7
<211> 16
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Peptide based on the wild type tobacco

mosaic virus protein

<400> 7
Cys Gly Ser Tyr Asn Arg Gly Ser Phe Ser Gln Ser Ser Gly Leu Val
1 5 10 15

<210> 8
<211> 16
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Peptide based on the wild type tobacco mosaic virus protein

<400> 8
Cys Gly Ser Tyr Asn Arg Phe Ser Phe Gly Arg Ser Ser Gly Leu Val
1 5 10 15

<210> 9
<211> 16
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Peptide based on the wild type tobacco mosaic virus protein

<400> 9
Cys Gly Ser Tyr Asn Arg Gln Ser Phe Asp Phe Ser Ser Gly Leu Val
1 5 10 15

<210> 10
<211> 16
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Peptide based on the wild type tobacco mosaic virus protein

<400> 10
Cys Gly Ser Tyr Asn Arg Arg Ser Phe Asp Gly Ser Ser Gly Leu Val
1 5 10 15

<210> 11
<211> 16
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Peptide based on the wild type tobacco mosaic virus protein

<400> 11

Cys Gly Ser Tyr Asn Arg Asp Ser Phe Arg Lys Ser Ser Gly Leu Val
1 5 10 15

<210> 12
<211> 16
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Peptide based on the wild type tobacco mosaic virus protein

<400> 12
Cys Gly Ser Tyr Asn Arg Asp Ser Phe Ser Ala Ser Ser Gly Leu Val
1 5 10 15

<210> 13
<211> 19
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Peptide based on the wild type tobacco mosaic virus protein

<400> 13
Cys Arg Gly Thr Gly Ser Tyr Asn Arg Asn Ser Phe Glu Ser Ser Ser
1 5 10 15
Gly Leu Val

<210> 14
<211> 19
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Peptide based on the wild type tobacco mosaic virus protein

<400> 14
Cys Arg Gly Thr Gly Ser Tyr Asn Arg Ser Ser Phe Glu Ala Ser Ser
1 5 10 15
Gly Leu Val

<210> 15
<211> 19
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Peptide based on the wild type tobacco mosaic virus protein

<400> 15
Cys Arg Gly Thr Gly Ser Tyr Asn Arg Ser Ser Phe Ala Ser Ser Ser
1 5 10 15
Gly Leu Val

INTERNATIONAL SEARCH REPORT

Inter ›nal Application No
PCT/SE 99/00921

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N27/327 G01N33/543 C12Q1/00 G06F19/00 //G06F159:00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE CHEMABS 'Online! CHEMICAL ABSTRACTS SERVICE, COLUMBUS, OHIO, US YAGISHITA, AKIO ET AL: "Regeneration of biosensor" retrieved from STN Database accession no. 111:74342 XP002117046 abstract & JP 63 229359 A (FUJITSU LTD., JAPAN) 26 September 1988 (1988-09-26) --- -/--	1,38

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 September 1999	13/10/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Moreno, C

1

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter 'onal Application No
PCI/SE 99/00921

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,X	DATABASE CHEMABS 'Online! CHEMICAL ABSTRACTS SERVICE, COLUMBUS, OHIO, US ANDERSSON, KARL ET AL: "Identification and Optimization of Regeneration Conditions for Affinity -Based Biosensor Assays. A Multivariate Cocktail Approach" retrieved from STN Database accession no. 131:85092 XP002117047 abstract & ANAL. CHEM. (1999), 71(13), 2475-2481 ,	1-50
P,X	WO 98 26288 A (BIACORE AB ;LOEFAAS STEFAN (SE)) 18 June 1998 (1998-06-18) the whole document	1,36,38, 40,50
A	EP 0 781 999 A (BEHRINGWERKE AG) 2 July 1997 (1997-07-02) the whole document	1,38
A	GB 2 270 976 A (MARCONI GEC LTD) 30 March 1994 (1994-03-30) examples	1,38

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

formation on patent family members

International Application No
PCT/SE 99/00921

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
JP 63229359 A	26-09-1988	NONE	
WO 9826288 A	18-06-1998	AU 7849798 A	03-07-1998
		EP 0944835 A	29-09-1999
EP 0781999 A	02-07-1997	DE 19548376 A	03-07-1997
		AU 7549896 A	03-07-1997
		CA 2193318 A	28-06-1997
		JP 9189701 A	22-07-1997
GB 2270976 A	30-03-1994	EP 0653065 A	17-05-1995
		EP 0660935 A	05-07-1995
		EP 0660933 A	05-07-1995
		WO 9403807 A	17-02-1994
		WO 9403811 A	17-02-1994
		WO 9407140 A	31-03-1994
		GB 2270158 A,B	02-03-1994
		GB 2269896 A,B	23-02-1994
		GB 2271634 A,B	20-04-1994
		US 5723304 A	03-03-1998